CONNECTIVE TISSUES

Transactions of the Fourth Conference
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Edited by

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ERRATA

Transactions of the Third Conference

1. Legend for Figure 1 should read.
   Kidney x 325. Basement membranes of tubules and glomerular vessels in red, reticulum in blue, basal cytoplasm in greenish yellow, brush border in purplish red, plasma in orange, erythrocytes in yellow
2. Legend for Figure 2 should read:
   Spleen x 720. Lattice fibers about sinuses in red, reticulum and connective tissue in blue, erythrocytes bright yellow, nuclei brown.

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Fourth Conference on Connective Tissues

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Fourth Conference on Connective Tissues

THE JOSIAH MACY, JR. FOUNDATION CONFERENCE PROGRAM

As AN INTRODUCTION to these Transactions of the Fourth Conference on Connective Tissues, I should like to outline what it is that the Foundation hopes to accomplish by its Conference Program. We are interested, first of all, in furthering knowledge about connective tissues, and to this end the participants were brought together to exchange ideas, experiences, data, and methods. In addition to this particular goal, however, there is a further, and perhaps more fundamental, aim which is shared by all our conference groups. This is the promotion of meaningful communication between scientific disciplines.

The problem of communication between disciplines we feel to be a very real and urgent one, the most effective advancement of the whole of science being to a large extent dependent upon it. Because of the accelerating rate at which new knowledge is accumulating, and because discoveries in one field so often result from information gained in quite another, channels must be established for the most effective dissemination and exchange of this knowledge.

The increasing realization that nature itself recognizes no boundaries makes it evident that the continued isolation of the several branches of science is a serious obstacle to scientific progress. Particularly is it true in medicine that the limited view through the lens of one discipline is no longer enough. For example, today medicine must be well versed in nuclear physics because of the tracer techniques and the injury which can result from radiation. At the other extreme, medicine is certainly a social science and, through mental health, must be concerned with economic and social questions. The answer, then, is not further fragmentation into increasingly isolated specialties, disciplines, and departments, but the integration of science and scientific knowledge for the enrichment of all branches. This integration, we feel, can be encouraged by providing opportunities for a multiprofessional approach to given topics.

Although the fertility of the multiprofessional approach is recognized, adequate provision is not made for it by our universities, scientific societies, or journals. And perhaps the presence of other

hindering factors must be admitted. Partly semantic in nature, they may also to some degree be psychological. Admittedly, it is oftentimes difficult to accept data derived from methods with which one is unfamiliar By making free and informal discussion the central core of our meetings, we hope to achieve an atmosphere which minimizes as much as possible these semantic and emotional barriers.

Thus, our conferences are in contrast to the usual scientific gatherings. Presentations are designed not to present neat solutions to tidy problems, but rather to elicit provocative discussion of the difficulties which are being encountered in research and practice We ask that the presentations be relatively brief, and emphasis is placed upon discussion as the heart of the meeting. Our hope is that the participants will come prepared not to defend a single point of view but, with open minds, to take full advantage of the meeting as an opportunity to speak with representatives of other disciplines in much the same way as they talk with their colleagues in their own laboratories.

During 1953, under the Conference Program, conferences will be held on the following topics· administrative medicine, adrenal cortex, aging, consciousness, cybernetics, infancy and childhood, liver injury, metabolic interrelations, nerve impulse, renal function, and shock and circulatory homeostasis

When a new conference is organized, the Chairman, in consultation with the Foundation, selects fifteen scientists to be the nucleus of the group which will hold annual meetings for a period of five years Every effort is made to include representatives from all pertinent disciplines From time to time, however, new members are added by the group to fill gaps in viewpoint or technique. A small number of guests is invited to attend each meeting, but, for the purposes of promoting full participation by all members and guests, attendance at any meeting is limited to twenty-five. During a conference's prescribed lifetime we cannot possibly include more than a small fraction of the key investigators in the field, and one of the difficulties in forming a group is that it is necessary to exclude so many investigators we should like to include

The transactions of these meetings are recorded and published. This is done because the Foundation wishes to make current thinking in a field available to all those working in it, and to those in other fields who are concerned with science: for example, government officials, administrators, etc.

in order to rearrange, to test, and to validate, research thrives on creativity which has its source in unconscious, nonrational processes Unfortunately, however, in the research reports which are presented to the world in scientific journals, this integral part of scientific endeavor is shriveled by the cold, white light of logic. By preserving the informality of our conferences in the published transactions, we hope to portray more accurately how the minds of scientists work and to give a truer picture of the role which creativity plays in scientific research.

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INTRODUCTORY REMARKS

Holbrook: Dr. Fremont-Smith has asked me, a number of times, how certain ones of us became interested in connective tissues from the devious paths by which we have approached it. He suggested that each of us take a minute or two and tell why and how we became interested. After all, we are pathologists, histologists, chemists, pharmacologists, and so forth. How is it that such a diversified group of people is concerned about connective tissues?

D. MURRAY ANGEVINE: As often happens, I came into this field accidentally. I had been interested in infection and immunity for some time Later, because of that, I was asked if I would work on the problem of rheumatic disease, which I did for a number of years. There was considerable money available to support this research However, I think specifically my interest in connective tissue stems from the time when a young doctor named Altschuler visited me and asked me what I thought fibrinoid was When I gave him my definition he said, "That doesn't seem right to me." We had quite a discussion, and when we got through, he said, "Dr. Angevine, I really came over to see about a residency in pathology. If I obtain such a residency, I should like to start a study on the nature of fibrinoid with you." He stayed for three years, and we worked on the problem, he doing most of the work. Thus, it was through a young man that my interest in it was aroused, rather than through any effort I had made on my part. He came to me, however, because of my work in rheumatic diseases.

Holbrook: That is very interesting Dr. Travell?

JANET TRAVELL: I came into this field, I think, by a most devious route When I graduated from medical school, I was primarily concerned with cardiology, and started working in clinics and hospitals in that field I became more and more interested in the pharmacology of cardiac drugs, which led me to Cornell. Then I went on into the field of cardiac pain. I found that many people with chest and arm pain had heart disease, some had lung disease, and some had no disease at all, so I became interested in the

interrelations of visceral pain and the somatic components of pain. From there, I moved on to the study of the mysterious abnormal states known as myofascial trigger areas, which are located in the tendons and muscles and fibrous structures, and apparently represent a generalized type of physiological reaction of these tissues to injury or to noxious stimuli of any kind That led me back to the problem of what actually goes on in the connective tissue itself

Holbrook. Dr. Meyer, what do the chemists see in this?

KARL MEYER: My interest came about in a devious way, too. I started rather accidentally I got a job, in 1933, with the Eye Institute, the ophthalmological institute of Columbia. The Institute had just opened and most of us didn't know what we should work on in ophthalmology, so the bacteriologist, Dr Thompson, and I decided we would work on lysozyme. We studied the mechanism of lysozyme, and found that the mechanism of this enzyme is the hydrolysis of a substance which we called "of mucoid nature" In those days we didn't actually have a definition of what it was There seemed to be a specific enzyme which we thought hydrolyzed an hexosamine-containing substance All we knew about mucoids was that they should contain hexosamine I then looked for substrates, or what the specificity of this enzyme might be, and tried to repeat Levine's work on the isolation of mucoitin-sulfuric acid from vitreous humor and from umbilical cord Those were the two sources which seemed to me to be the best The vitreous was directly connected with the eye We found very shortly that there was nothing there, as Levine had said We found hyaluronic acid there, but even then, for years my interest was with the hexosamine-containing substance rather than with connective tissue, until we realized that this is a rather broad area of interest from the chemical point of view It is something which is easier to deal with than the more complex substances, such as the blood, with which I had come in contact. So I got into connective tissue

Holbrook. Dr Fell, would you care to tell us how you became interested in connective tissues?

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one of the organs that has the most interesting developmental mechanics is the skeleton. I have worked on a number of other tissues, but I seem always to come back to the skeleton in the end. I think the thing that fascinates me about it is that during its earlier stages, the whole thing develops to a great extent as a mosaic. Its gross architecture is determined very early, and then it unfolds itself like one of those Japanese flowers in water. Later on, when the skeleton is ossified, you have this rigid structure which is, in fact, probably the most plastic tissue in the body, and this paradoxical state of affairs has always intrigued me very much. I think that is why I have always reverted to it in spite of excursions into other tissues.

Holbrook. Dr. Dempsey?

EDWARD W. DEMPSEY: There are two reasons that I can advance, but I am not sure that they have any resemblance to reality. They are the rationalizations that I come up with at the moment One of them is that early in my scientific life I was interested, and still am, in endocrinology, and it suddenly occurred to me that we attempted to explain all endocrine phenomena in terms of a hormone acting on a target organ, and considered the target organ in all cases to be the parenchymatous tissue of that organ. The thyrotropic hormone, for example, was thought to act on the thyroid cell, and secondarily the thyroid cell caused an increase in blood supply and an increased interchange. The circulatory or connective tissue effects therefore were secondary. It seemed to me we had by no means ruled out the possibility that the circulatory and connective tissue changes in transfer might not be primary. That is, the tropic hormone might act on the vascular system and connective tissue, modifying the barrier between vascular system and cell. I have never been able completely to get rid of this idea nor to devise a way of critically testing it. Then, I suppose the other reason I am interested in the connective tissues is that I am cursed with the kind of mind that refuses to stick to one discrete and special topic, and no matter how I try to hold my attention to one restricted area, I find that it spreads out to the surrounding information. As a histologist, I can't keep my eye exclusively upon the parenchymatous cells of an organ, but I begin to think about what that cell rests on and

what is contiguous to it. Such reflections have indicated to me that we know painfully little about the connective tissues which form the framework of all the rest of the cells in the body.

KEITH R. PORTER: As you can see, my career in connective tissues has been extremely short. I think that anything I could now remember as leading me into the study of this subject would not prove very interesting I might say I have in a general way been interested in the origin of tissue components, i e., their morphogenesis, and with the techniques we had available, the morphogenesis of collagen seemed readily studied, so we looked into it.

GRANVILLE A. BENNETT: My interest probably resulted from environmental circumstances In the first place, a pathologist probably sees more connective tissue under the microscope than any other type of tissue More particularly, however, I had my training under Dr S Burt Wolbach For a long time he had been interested in connective tissue and the effects of certain vitamins on formation and maintenance of intercellular substances His interests doubtlessly influenced most of us who were associated with him An opportunity was afforded me to become a member of an investigative team for the study of crippling disease, and, of course, crippling disease led us directly into problems of the connective tissues. Thus, circumstances of environment, mostly accidental, were determinants in my case.

Holbrook I suspect it was with most of us. Dr Hass would you care to say how in the world you got into this muscle business, for instance?

GEORGE M. HASS: Our interest in muscle, I think, arose basically as a consequence of our inability as pathologists to explain cardiac failure in human beings The customary explanation which pathologists give, and which I have always given for most cases of human cardiac failure, is that it is due to a deficiency in the blood supply to the myocardium If there is no deficiency in the blood supply to the myocardium, which can be adequately shown, the usual explanation of the failure of the heart is on the basis of a few minute lesions which may occur here and there throughout the muscle It happened that prior to the beginning of this particular investigation Dr Bennett, and I, had trained under Dr Wolbach in pathology and

I wish that I could have had more of it. The connective tissues were among Dr. Wolbach's many and varied interests. His contributions to an understanding of the relationship between certain vitamins and the development of the interstitial tissues continue to be models of scientific accuracy and penetrating perspective. As a result of his encouragement, some of the first work that I did was on the chemical composition of amyloid and cartilage. That was followed by some studies on the genesis of collagen *in vitro,* and some work on the isolation of elastic tissue in pure form with characterization of its physical properties But this present investigation was excited largely by our interest in why the human heart failed.

Holbrook Dr Fremont-Smith?

FRANK FREMONT-SMITH· My first interest in connective tissues was in second-year medical school when I began to get a chance to look at sections of experimental wound healing, and was amazed at the connective tissue response I also was very much influenced by Wolbach, and had a year of pathology with him in Boston at the Peter Bent Brigham Hospital just after I got through medical school I remember how much his attempts to make a differential diagnosis impressed me. He also focused on the connective tissue stroma, and I knew then that there was something about connective tissue which was very important. However, I never went much further with it In our Liver Injury Conference, we had a special subcommittee of ten or fifteen pathologists, who were trying to agree on the nature of cirrhosis of the liver I found, then, what happens when you try to get any ten pathologists together to agree even on sections from the same liver It was at that time that I learned somehing about connective tissue and about agreements and disagreements concerning it.

Holbrook: Dr Ragan?

CHARLES RAGAN: My interest in connective tissues evolved from an interest in the clinical problems of arthritis and the study of synovial fluid. This fluid may loosely be considered a type of ground substance. My chief interest now is in the study of simple inflammatory processes occurring in this tissue

W. PAUL HOLBROOK: That is very interesting. I came in rather by the back door, too. I started in chemistry as a young man,

and then went to internal medicine and cardiology rather accidentally, thinking nothing at all much about connective tissues or their diseases My confreres at the hospital began to send me patients who had various forms of rheumatic disease, largely chronic arthritis, for a cardiac consultation You all know the story: over and over again nothing clinical could be found and repeatedly I would report, "There is nothing the matter with this man's heart that I can find, why don't you do something about his arthritis?" The answer was always, "Well, what do you do about arthritis?" and my invariable response was, "I don't have any idea, but don't you know?" After that had gone on for a while I became intensely curious about arthritis, since nobody seemed to know what to do about it As most of you remember, twenty-five years ago we didn't even know the names of the various types it was just chronic arthritis From that experience, largely out of curiosity, I began to orient my thinking toward connective tissues.

SUGGESTED OUTLINE FOR DISCUSSION OF AREAS OF AGREEMENT

Structure may be investigated by

- I. Light microscope
- II Electron microscope
- III X-ray diffraction
- IV Study of chemical composition by.
  - a) Isolation techniques
  - b) Histochemical techniques

Dynamic processes may be explored through

- I Histochemical techniques
- II Observation during growth and maturation
- III Observation of responses to noxious stimuli
- IV Tissue culture techniques

Connective tissue may be divided as follows.

Normal structure

- I Cellular
  - a) Fibroblast [1, 2]
  - b) Macrophage [1, 2]
  - c) Mast cell [1, 2]
- II Fibrillar
  - a) Collagen [3, 4]
    - 1 Physical characteristics
    - 2 Chemical composition
    - 3 Orientation
    - 4 Turnover
  - b) Reticulin [5]
  - c) Elastic fibers
    - 1 Physical structure
    - 2 Chemical composition
    - 3 Derivation
    - 4 Turnover
    - 5 Changes with age and trauma
- III Interfibrillar material "ground substance" [6, 7]
  - a) Mucopolysaccharide component
    - 1 Distribution
    - 2 Chemical composition [8]
  - b) Protein components – unknown

Abnormal states

- I Fibrinoid [9]
- II Collagen and ground substances in disease [10]
- III Responses to trauma –
  - a) Local
  - b) Antibodies

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[1] The precursor of these cells is a matter of dispute

[2] The life cycle of these cells is a matter of dispute.

[3] Probably secreted by the fibroblast.

GENERAL AREAS OF AGREEMENT REACHED IN THIS CONFERENCE GROUP

INFORMAL GROUP INTERCHANGE

Holbrook Quite some time ago I asked Dr Ragan if, as Editor of the Transactions of this Conference group on Connective Tissues, he would be willing to spend a few minutes reviewing those areas in which the group had reached agreement during the three previous annual meetings, and focusing attention upon those areas where there was still disagreement among the members. He consented to do this Dr Ragan will you start the discussion?

Ragan As Dr Holbrook has said, he has asked me to make an informal outline concerning general areas of agreement reached in the three conferences we have held in the past I have compiled an outline and the areas of agreement are very few, however, after a little discussion we might possibly add more There are undoubtedly additional points which will occur to each of you, and if you could add them to the outline we would have at least some sort of syllabus of our past efforts and, in broad general terms, what is still awaiting further study

I have tried to include the disciplines involved in these conferences, and in the field of connective tissues in general, divided into three categories structure, chemical composition, and dynamic processes Those concerned with structure may be examined at the resolution of the light microscope, and at higher resolutions The disciplines concerning chemical composition may be subdivided into isolation and histochemical techniques Lastly, dynamic processes, (in which histochemical techniques may be used) include the study of growth and maturation, responses to noxious stimuli, and the reaction of the various tissues in culture Do you feel this is a fair statement?

[4] Definition — see *Metabolic Interrelations* Reifenstein, Edward C, Jr, Editor Trans Fourth Conf New York, Josiah Macy, Jr Foundation, 1952 (pp 72-73)
[5] Dispute continues regarding the identity of collagen and reticulin
[6] The particular cell which secretes the ground substance is a matter of dispute fibroblast (Gersh), mast cell (Asboe-Hansen)
[7] Theories concerning the protein-polysaccharide linkages are varied and in very few instances even proposed
[8] Hyaluronic acid and the chondroitin sulfates A, B and C have been isolated to date
[9] The composition and derivation of fibrinoid remain disputable
[10] There are widely divergent views concerning changes in collagen and in ground substance in various disease states

Angevine: I wonder whether it wouldn't be better, Dr. Ragan, to omit the histochemical techniques from one section? I realize that they represent dynamic processes, but I think if you leave them under the chemical processes it might be clearer.

Ragan. I agree, *dynamic processes may be studied* by any or all of the disciplines involved

Meyer: One can imagine that there are some dynamic processes which should be studied by histological techniques, also. Instead of this division into three areas, I prefer two subdivisions: namely, the analysis of structure and of function, which I think really covers it The analysis of structure is the analysis both of the physical and chemical structure.

Holbrook: If the Chairman might say a word, it seems to me that Dr. Ragan has one point, that chemical composition may not necessarily show us anything about dynamics

Travell. Or about structure.

Fremont-Smith: We seem to be trying to find, on the one hand, a logical classification, and on the other a classification that bears some relationship to reality. The two cannot take place simultaneously Some of us prefer logic, some of us prefer reality, and some of us prefer to take a path somewhere between the two. I think that really is part of the issue.

Travell. The distinction is between something that is static as you look at it, both in structure and chemistry, as contrasted with dynamic function

Dempsey: I thought we agreed, very early in the conference, that when we talked about composition, we meant structural and chemical composition, without making any sharp differentiation between them From the standpoint of a working classification, though, I think it is good not to distinguish too sharply as yet.

Ragan For a working classification, "concerning chemical composition," it might suffice.

Travell. I think that would be fine.

Angevine. Yes, that would cover it.

Dempsey: I agree.

Fremont-Smith: It would be a definite advantage to do so

Ragan Does the next category meet with your approval? Do you think connective tissue might be divided into three components: cellular, fibrillar, and interfibrillar?

Angevine: I would agree with that.

Ragan. Do you think there is any point in making the distinction between fibrillar and interfibrillar?

Dempsey Yes, I like the word "interfibrillar" much better than a word which would try to designate what it is. "ground substance," for instance

Fremont-Smith: I was going to say "other," but I am very happy with "interfibrillar"

Holbrook. I like it very much

Ragan Next, we must consider two categories normal structure and abnormal states There is adequate reason to doubt that this is proper but, for the sake of argument, let's go ahead Under normal structure, cellular component, only fibroblasts and macrophages are included I should probably have included some other cells. Does anyone want to include mast cells? Do you think it makes any difference?

Angevine. What do you mean by "macrophage"? I don't want to get into the problem of definition, but it is considered by some that the macrophage may change to, or arise from, the fibroblast I should prefer just to leave the word fibroblast and not say much about the macrophage and perhaps add the mast cell, at least where we are dealing with the cellular structure, because although there are macrophages in connective tissue, they are also everywhere in the body – in every organ Dr Dempsey may disagree with this, but I don't think that the macrophage has much to do with the function, or the normal structure, of connective tissue The macrophage cell is in the brain, in the kidney, in the spleen, and in lymph nodes it is everywhere I think if we just lifted the macrophage system out of this it might simplify our study of connective tissue Would you agree with that, Dr Ragan?

Ragan At the Second Conference, we went into this question in great detail

Fremont-Smith. Suppose you just used the word "precursors" and didn't go beyond that?

Angevine That would be fair enough I realize that the macrophage is an integral part of this structure, but the macrophage system, in my conception, isn't very active unless it has something to do Activity takes place when there is injury

Bennett Could we agree on the designation "mesenchymal cells" – differentiated and nondifferentiated?

Dempsey May I enter into this? My objection is not really based on whether or not the macrophage is a derivative of, or related to, the fibroblast However, if we are talking about the normal structure of connective tissue, I don't think it can be isolated to merely one cell or two cells, because the connective tissues are a group

of tissues recognized by histologists Out of their disagreements expressed over many years, there has come to be a general kind of agreement that in connective tissues one comes across cells that are called fibroblasts, macrophages, eosinophils, mast cells, lymphocytes, and other cellular elements I don't think we can abstract the connective tissues yet to a single cell and its products. If the macrophage is removed from this classification, all that is left is the fibroblast, implying that anything else is adventitious and extraneous. I don't think we are sure of that, so I don't think we can really improve, at the moment, on the histological classification of the cellular elements of the connective tissues.

Angevine: If you wish to put in the term "mesenchymal cell," all right. However, as soon as the term "macrophage" is included, you have passed from normal structure into pathology, and if it is assumed that there is a phagocytic system in the body, whatever this large phagocytic cell is called, it will not phagocytose unless there is disease or some other process taking place. Therefore, why not just call it a mesenchymal cell? Of course, that includes a fibroblast, too Or call it a tissue monocyte or, whatever you wish, but the macrophage does suggest phagocytosis

Dempsey I think the point Dr Angevine is trying to make here is that phagocytosis is a physiological concept. You are trying to identify a cell which can do something physiological by structural means, and that can't be done

Angevine· That is exactly the point

Dempsey: A phagocyte is not a phagocyte until it has something to eat.

Fremont-Smith: But dead cells are also normal. They are not normal as cells, but it is normal for them to be present in the living organism, even in the embryo Dead glomeruli for instance, appear at least by birth, so that there is in the living organism the greatest abnormality we know of. dead tissues which have to be phagocytosed. Therefore, it seems to me one can say that the normal organism may have abnormal parts to it.

Dempsey I think nobody would describe any of the people around this table as murderers, yet any one of us might commit murder under certain circumstances. You can't describe the cell that you see under the microscope as a phagocyte until you see that it has eaten something.

Fremont-Smith· That's right.

Dempsey· It is a different test.

Bennett: Not only that, but mesenchymal cells among cell populations may be highly differentiated for example, the mesenchymal cells, which line synovial clefts, can acquire phagocytic properties. Under certain abnormal circumstances they may accumulate quantities of siderotic pigment and lipid within their cytoplasm Hence, I think there is a connotation of function in the word "phagocytosis," which must be recognized in classifying connective tissue cells

Angevine. The term, I believe, was originally mentioned by Metchnikoff (1) in connection with inflammation, he described two types of cells, a macrophage and a microphage The former was the mononuclear cell, and the latter was the polymorphonuclear. I am not quarreling with terminology or trying to force it, but if people read about normal structure and see "macrophage," they immediately come up against the same problem which we are struggling with, and I wonder if we used .

Ragan: Histocyte?

Angevine: Yes, histocyte would be more satisfactory to me. I'll accept anything Dr Dempsey says except that I should like to get the "phage" out of it

Ragan What do you prefer, Dr Dempsey?

Dempsey I should like to get rid of the term, "normal structure"

Ragan Do you want to say fibroblast, mast cell, and wandering cell?

Dempsey. You have got to put in the lymphocytes and the polys, too, because they are normal in the connective tissues, at least in some of them

Meyer And how about the cartilage? Is that a derived cell, or doesn't it come in here?

Fell: And osteoblasts

Ragan Are you willing, Dr Fell, to call them fibroblasts?

Fell No

Bennett: I don't wish to belabor the point, but is there any merit in the initial suggestion I made, of speaking of them as mesenchymal cells, differentiated and nondifferentiated?

Fremont-Smith I should think so

Bennett I think that is the best alternative Of course we then find ourselves in the dilemma of dealing with muscle.

Dempsey We're in a dilemma, anyway

Ragan We don't need to itemize differentiated or nondifferentiated mesenchymal cells

Bennett I think that is the best way out of it; it is inclusive

Ragan: All right. I think this is begging the issue, more or less. We do have to talk about the origin and the turnover of each.

Meyer: Oh, yes

Ragan: Then we will call them mesenchymal cells, differentiated and nondifferentiated, and the origin and turnover of each.

Travell. Are we going to list any of the cells under these two classifications?

Ragan: The group doesn't seem to want to

Fremont-Smith: They're afraid. When we give them names we have an argument.

Travell: Dr. Ragan says we are begging the issue.

Fremont-Smith: He's right

Angevine: I think Dr Ragan would be happy if we just said "mesenchymal" cells, wouldn't he?

Ragan: No, I think we have to specify each differentiated and nondifferentiated cell. The next part of my outline concerns fibrillar components.

Angevine: This may sound very academic to this group, but I am sure that Dr Dempsey, who teaches histology, and I, who teach pathology, find that this is one of the most difficult problems to present to medical students at the first- and second-year levels

Fremont-Smith: Which – the fibrillar or the mesenchymal?

Angevine· No, giving the student a clear conception of this whole phagocytic system in the body is difficult in view of the conflicting terminology used in various texts.

Ragan Could it be that we don't know anything about it? That is why it is difficult to explain?

Angevine: No, I think we know a good deal about it, but I think that we are probably speaking different languages

Ragan: Collagen once laid down, evidently stays there for a long while We have an idea of the life span of many cells, but not of the fibroblast.

Travell. In a test tube collagen fibers can be dissolved and precipitated, reformed and redissolved How do we know but that that goes on in living organism?

Ragan We certainly don't know whether it does or does not

Meyer· Work has been done on formation of collagen Normally it seems to be a very slow process I must admit that we know very little about it, though

Fremont-Smith· May I point out that whenever a group of people from several different disciplines and points of view try to find their areas of agreement, and finally put them down, it is the most unin-

teresting possible statement and practically meaningless. The only areas which are of interest and significance are the areas on which they are working· these are the ones on which they have divergent views, and unfortunately have to be eliminated in order to find a common area of agreement I have seen that happen before. From time to time, people feel the need to say what they agree upon, whereas, from my point of view, the things they do not agree upon are so much more interesting In other words, to agree upon the specifications of our disagreements is a very exciting thing to do There you have a dynamic process The moment you do it, you immediately see new experiments which must be done, and things move on.

Dempsey: I agree with what Dr Fremont-Smith says and I think I can amplify it, perhaps. There are three experimental techniques that are involved in what we are debating One is morphological the pure structure of these cells, as one can see them in sections or spreads of connective tissues examined with the microscope The next one is physiological what the cell is able to do, and whether it can or can not ingest foreign material This is a quite separate experimental problem The third one is embryological: what a given cell can turn into, under suitable circumstances

Fremont-Smith. What its life history is

Dempsey. Yes, and whether what we see structurally in one stage of its career is the equivalent of what we see at another phase, in other words, whether we could recognize the old man from the picture of the baby, and vice versa.

Now, these are phenomenonological difficulties We have a perfectly straightforward set of phenomena, involving structure, function, dynamics, and change of state with time As long as we stick to any one of those concepts, we are reasonably clear As soon as we begin to try to transpose information gained from one procedure to another, then we get into trouble That is where Dr Angevine stumbles on the word, "phagocytosis," because he is not sure he can look at a cell under a microscope and tell whether or not it is a phagocyte Isn't that right?

Angevine. That is right, unless the cell has phagocytosed something

Dempsey· Unless it has trypan blue in it.

Angevine: I realize that many of these cells are potentially phagocytic, but you cannot tell that under the microscope.

Dempsey You can't tell unless you do the experiment to see

which one is and which is not, so what we are doing is confusing one set of observations with another set.

Fremont-Smith: But not confusing our issue. It seems to me that when we use the word "confusing" here, we are confusing ourselves, and that the only hope of progress in this field is to try to correlate these different forms of observation. This is a difficult problem It is only in that sense that I object to the word confusing, as if it were something we should not do

Dempsey: Oh, no!

Fremont-Smith. And it seems to me that there we shall not reach agreement, or if we do, we shall reach it only on the least interesting part.

Porter I wonder where connective tissue begins and where it ends? Where you have collagen, your cell population might be better described as multiple, and let it go at that. There is collagen [illegible] just under the basement membrane [illegible] e latter does not suggest that fibroblasts put it there. [illegible] lagen in cartilage and bone, and cartilage cells put it down, apparently. Perhaps we should not attempt to describe the cellular elements involved, and say there is everything except epithelial cells?

Fell Do we know that epithelial cells don't form collagen? I have a shrewd suspicion that they do.

Dempsey. There is at least one place where these mesodermal cells don't lay down collagen and that is in the blood. There isn't any collagen floating around inside our blood vessels.

Fremont-Smith. At least we don't see it.

Dempsey: So we have to include fibrin and fibrinogen, don't we?

Fremont-Smith. Are we absolutely sure?

Angevine. I think I would bet on that; that is one thing I think I could be sure of.

Dempsey: Nobody is ever sure of anything.

Fremont-Smith: They weren't picked out by the liver

Travell: Nobody disputes it

Fremont-Smith: All right; we will accept it for the moment.

Bennett Let us go back just a moment to the comment Dr. Angevine made, about the difficulties of teaching students. Dr. King in our department this year, who is trying to reconcile in the minds of the students the differences between anatomists, histologists, and pathologists, made what I thought was a rather clear-cut observation. He pointed out that of the tissues that we call mesodermal, there are certain predestined cells that go into organized

structures, such as capillaries, and endothelium-lined and mesodermal-lined spaces After all these substances have been exhausted and taken out, there remain mesenchymal elements that are capable of forming collagen, whether they are cartilage cells, fibroblasts, or osteoblasts They have the capacity of elaborating in some way other types of intercellular substances In addition there is the cell population, which has not differentiated beyond a stage in which it could revert a little, or perhaps progress a little, towards becoming potentially phagocytic cells, under appropriate stimuli, and also do other things I think perhaps it might be well to look upon this tissue as one of the elements of the mesoderm from which there have been extracted predestined cellular functional patterns of cells that form certain organized structures Then the rest of it is residue, which at any time may pick up the trend and develop into things that normally are developed out of mesoderm

Fremont-Smith. It may develop new predestinations?

Bennett Yes, that is possibly so

Holbrook We have talked rather glibly about all the mesenchymal cells producing collagen, but do you know of any evidence, Dr Porter, via the electron microscope, or otherwise, that this is so? Have you seen any cells other than the fibroblasts which you are sure make collagen?

Porter: No, I am sure the fibroblast does, but I am not sure what the others do.

Holbrook All of us are convinced that the fibroblast does

Fell: An osteoblast does. Probably a chondroblast also

Ragan. Can you be sure that a chondroblast stays a chondroblast while it is making collagen?

Fell. It looks like a chondroblast

Fremont-Smith: It depends on how you define it

Holbrook. There isn't any possibility that some of the collagen is taken from solution and reconverted?

Porter Possibly so, in the case of the collagen in the sarcolemma and under the basement membrane

Holbrook. The exact identification of the fibroblast which makes collagen, is not so essential as that we demonstrate that each single mesenchymal cell can in itself produce collagen, because I can conceive of some collagen, certainly, as being reconstituted from solution

Dempsey This is almost the same thing that is called "complementarianism" The word, I think, is derived from the concepts dealing with light as a particle, and light as a wave Finally the

physicists decided it was a poor argument and said, "When you look at it one way, it's a wave, and when you look at it another way, it's a particle, and each one of the concepts complements the other one. The truth is that it is the sum of both, and the method that one applies in one's experiment determines the answer that one is going to get."

Fremont-Smith: Yes, on the whole.

Dempsey: We are dealing here with the same kind of thing A chondroblast is a fibroblast because it lays down collagen When we look at it one way, it is a cell which is different when we see it from another aspect. The entire truth about this cell is something that transcends any single one of the techniques we apply.

Fremont-Smith: Or that transcends the sum of all the techniques that we now use?

Dempsey: I think so, yes.

Angevine· Would you feel, Dr. Dempsey, that when we are discussing connective tissue, we probably have to discuss either areolar connective tissue, or one of the specialized forms of connective tissue separately? In other words, if we throw everything into one group, we get into difficulty. I think I agree with what Dr. Dempsey said, but I gather that most of the things we are discussing now on the basis of this outline relate to the areolar connective tissues, with a few exceptions such as in the specialized tissues as cartilage, bone or fat There will be other things added, which I suspect are all potentially capable of similar functions, and even revert to similar states, but if we are going to define something, we should first define areolar connective tissue and proceed from there. That would be logical, wouldn't it?

Ragan: I thought we had one area of agreement: that collagen was derived from a fibroblast.

Holbrook. I agree.

Ragan: But Dr Fell feels osteoblasts, chondroblasts, and so on, may lay down collagen. Should that be included?

Fremont-Smith. And also other unknown sources?

Dempsey: Perhaps we should say "other nondifferentiated sources" I think the only thing we agree on here is that we are dealing with connective tissues. Sometimes we are not even sure of that

Ragan: One thing we agreed on, before Dr. Fell came into it, was that collagen was derived from a cell.

Fremont-Smith· No, Dr. Porter wouldn't agree to that!

Ragan· Sarcolemma could have been laid down by a fibroblast.

Fremont-Smith. It could have been, but we are not sure of it

Angevine: Muscle is considered to be another one of these specialized connective tissues, also

Ragan: Dr. Fell, what do you think is the derivation of collagen?

Fell: Who knows!

Porter. Why not be satisfied with the fibroblast? We might even say that when a chondroblast is putting down collagen it is a fibroblast, later in its history it becomes a chondroblast

Ragan: Dr Fell doesn't agree with that

Fremont-Smith. Perhaps we can get Dr Fell to tell us something about this without narrowing it down to where she must say "Yes" or "No".

Fell· I am not quite clear as to what the question is now.

Fremont-Smith· What surprises you most about collagen?

Ragan. Dr. Fell, do you disagree with the statement I have outlined, that fibrillar collagen probably is derived from a fibroblast?

Fell. The only point I was trying to make was that bone has a dense network of typical collagen in it, and yet the collagen has other properties which differentiate it quite sharply from areolar tissue, it seems to me that the definition we had applied only to the areolar tissue There are, no doubt, other constituents in the bone that make it different in consistency.

Ragan· Would everybody feel happier if we called this conference the Conference on Areolar Tissue, or Loose Areolar Tissue?

Holbrook. No, no!

Angevine No, I wouldn't agree with that Actually, though, I think it has been areolar connective tissue that has been discussed to date. In the original talk you asked me to give at the First Conference, we said there were several specialized types of connective tissue, but we talked principally about areolar connective tissue, I think Dr Gersh, at the Second Conference, talked largely about areolar connective tissue, as did Dr Lillie and Dr Robb-Smith at the Third Conference Everybody here, of course, realizes that we have these other forms of specialized connective tissue My conception was that after the areolar tissues were fully discussed, we might branch out into the special tissues I think it would be wrong to call it all areolar connective tissue, but it is worth while to point out that that is what we have been talking about to date.

Travell. We talked about cartilage, the *symphysis pubis*, and other areas of the body We haven't actually limited the scope It is just that more information is available on areolar connective tissue

Angevine: Yes, but the *symphysis pubis* is not cartilage. It is *pure areolar connective tissue*.

Holbrook: Dr. Ragan, would it be complete if we said "and other mesenchymal cells"?

Ragan: I think it would be very complete if we said "normal structure of loose connective tissue."

Travell. But we don't want to limit it to loose connective tissue

Ragan. This is as far as we have gone.

Angevine. It would be better to say "areolar," which includes both loose and dense. If we are going to modify it at all, areolar would be more proper.

Dempsey: I think we are quibbling We are reaching the point where we are merely using words and I think none of us knows what he is talking about.

Ragan. Let's leave it as it is, then In the Transactions of the Fourth Conference on *Metabolic Interrelations* (2) they asked several people to give a definition of mammalian collagen Dr. Jerome Gross said "*Collagen* is a term embracing a class of fibrous proteins characterized by a particular wide-angle x-ray diffraction pattern, many members of the class exhibiting a fiber axis period averaging 640 Angström units. The known collagens of the higher animals are chemically characterized by a low content of *aromatic amino acids, and a high content of pyrrolidine* amino acids and glycine *Ground-substance* can be defined as the extracellular and interfibrillar amorphous component of all tissues"

Dr Paul B. Hamilton said. "*Collagen* – a term reserved for native fibrous proteins exhibiting specific spatial periodic wide-angle x-ray diffraction patterns, and containing from 6 to 7 per cent of total N as hydroxyproline N, from 10 to 12 per cent as proline N, 1 per cent as hydroxylysine N, and 25 per cent as glycine N."

Dr. Karl Meyer said: "The term *collagen* is ambiguous and is defined histologically (by microscopy in visible light and by submicroscopic techniques) or chemically. In the chemical definition, *the term should be reserved for material from mammalian sources* having a certain composition including the content of glycine, proline, and hydroxyproline, and hydrolyzed specifically by the enzyme, collagenase. All fibers of similar composition and histological appearance might be called *collagenoid*."

I thought it would be a good idea to use the term "collagenoid" and stop using collagen, because we can't come to any definition of collagen at this conference.

Meyer. When we hear that the chondroblast, as well as the fibroblast, lays down collagen, then the question does come up whether or not the various fibers are chemically identical or not. I do not know any evidence to the contrary, but there are great differences in solubility of collagen in different tissues I think the term collagenoid is a useful term.

Porter. To cover everything?

Meyer. Oh, no, it would not include everything

Fremont-Smith But several things

Meyer. Yes, it gives some leeway like the term "steroid," which means more than cholesterol – I believe the only sterol known at one time.

Fell: I should think it was very unlikely that the collagen was the same in different tissues When you watch osteogenic fibers being laid down in life, or in a living ossifying culture, it has quite a different appearance from the collagen fibers laid down in connective tissue.

Holbrook Dr Fell, does it have the periodicity?

Fell It has the same periodicity, but if you watch a living tissue culture that is laying down bone fibers, it has quite a different appearance from living areolar tissue that is laying down collagen fibers in culture. They seem much more rigid for one thing.

Fremont-Smith· Periodicity is only one form of measurement, and therefore there is nothing holy about it

Holbrook Oh, yes, I know that

Fell· After all, one knows, according to Parker's work (3,4), that fibroblasts from different places are physiologically different, so it is quite possible that their products may differ in some degree all over the body

Fremont-Smith. Isn't this a situation where the collagen fibrils would be both the same and yet different? That is, they would be the same in all parts of the body with respect to certain things, such as periodicity?

Fell Probably, yes.

Fremont-Smith And they would be different in one part of the body from every other, with respect to other characteristics

Fell. Possibly chemical, yes

Fremont-Smith There are similarities which would be very important with respect to certain problems, and their differences would be all important with respect to other problems. This is the kind of issue over which people will fight to the death to determine which is more important, the similarities or the differences, when

actually they are both of the utmost importance, but for different purposes. You see this kind of issue come up again and again in our conferences; people will say, "Is it the same or is it different?" when it is probably both.

Angevine. I should hate to see this term "collagenoid" accepted It is like "lymphomatosis" You are going to have to say, "elastoid" eventually, or "agglutinoid," or "albuminoid." Why not just say "collagen"? It has considerable variations in various sites. I don't think we are going to get anywhere by dropping the term "collagen," because one should realize that it may vary tremendously under many conditions. I think this would be retrogressive rather than a progressive step.

Fremont-Smith: It doesn't add to our knowledge.

Angevine: That is what I mean. If you can add something by changing a term, then it is all right.

Meyer: If you would call it "collagens," I would agree with you, but not the singular.

Angevine: I would agree on "collagens."

Fremont-Smith: Put it in the plural, then.

Angevine: I think that has been used.

Dempsey: Yes, it has been used, and it is quite useful

Ragan: Then it is "collagens."

Travell: That would include the collagen-like fibers seen in fish, with different chemical characteristics from the collagen seen in mammals.

Fremont-Smith: You can talk about mammalian collagens and nonmammalian collagens, which would include the fish.

Fell: I think whether you included a given collagen in a group would rather depend on whether there were more similarities, or more differences, between it and the group

Fremont-Smith: Or whether you were more concerned with the few similarities, or with the many differences, or vice versa.

Meyer: But if you call it mammalian, you do not think of one species. If you say "collagen" you think of it defined as chemically, physically, or histologically one and the same chemical compound. Unfortunately, this original histological concept has taken on a chemical concept.

Dempsey One thing which I think is missing from your definition, interestingly enough, is the original definition of collagen. The word means glue-forming, does it not? The original definition was on the basis of its solubility.

Meyer· Dr Lillie brought that out last year.

Dempsey: I am interested in going back to these definitions which Dr Ragan read, about that 7 per cent hydroxyproline N, and periodicity with the electron microscope. The one historical fact, which has differentiated collagen from any other substance in the body, was unfortunately omitted.

Travell: On hydrolysis it yields gelatin.

Ragan. We have quite a bit of data on the physical characteristics of collagen, and on the chemical composition of collagen. These data are factual and hardly warrant further discussion at this time. There is, however, the question of the orientation of collagen, which I think has not been solved

Meyer. I don't understand that

Travell: Meaning what?

Ragan: Why the fiber is laid down in a certain plane.

Porter. I would suggest the use of the word "organization."

Ragan· Yes, organization probably would be better

Travell: We are oriented, the fibers are organized

Ragan: I was thinking principally of Weiss' idea, with the three balls That is why I was thinking of orientation

Fremont-Smith: You are speaking of the pattern of several fibers in relation to one another, and not the pattern of a single fiber

Ragan. Why the fibril becomes a fiber and also why it is laid down in a certain direction That was my meaning

Porter· A good bit of that is certainly the product of tensions I know, as Dr. Fell does and everybody else who studies *in vitro*, that they tend to migrate along lines of stress, and that a fibroblast, placed under some tension, will develop intracellular cytoplasmic fibrils in response to that stress From what I have observed, collagen fibrils tend to be oriented along the same lines I suspect that as far as wandering fibroblasts are concerned, the stress factor is functional in controlling the orientation of the fibers

Ragan Suppose we say "along lines of stress?" The turnover of collagen is something which can probably be best studied in abnormal states I think Robertson's (5) data on the turnover in scurvy are about the best we have For instance, data of Sprinson and Rittenberg (6) on the turnover of the carcass protein showed it to be very slow. Perrone (7,8) also has shown that turnover is slow in adult animals, and in his data distinguished between "skeletal collagen" and "repair collagen"

Dempsey There is a word of caution which I should like to place in the record, if I may It seems to me that the experiments done on turnover of these fibers have been done in relatively static

physiological states. There has not been, so far as I know, any study on turnover done in situations in which one might expect the rapid laying down of collagen, or more importantly, the rapid destruction of collagen I don't think this is a closed issue. This is a place, Dr Fremont-Smith, where I think premature agreement, on the basis of our present data, would be too bad; I think more research is needed

Fremont-Smith: I agree with you; in fact, I am all for disagreement of this kind

Ragan I don't think we have enough on which to disagree, Dr. Fremont-Smith. This is complete ignorance.

Fremont-Smith: We can agree on our ignorance.

Ragan: Until the last conference, there was uniformity of opinion among the group that reticulum was identical with collagen.

Fremont-Smith. Had the issue been discussed thoroughly?

Ragan: It had been discussed on numerous occasions and, at the last conference Dr. Robb-Smith was very definite when he said he did not think they were identical. I think that is as far as we can go.

Meyer: A paper on reticulin has been published recently in *Nature* (9) in which the authors, Little and Kramer, state that *reticulin is distinct from collagen*. The midcortical connective tissue of the kidneys of children free from renal disease was found to consist almost entirely of reticulin. They reported that the collagen in this region occurs in the immediate neighborhood of larger blood vessels.

In the electron microscope, unshadowed specimens appeared as very thin laminated membranes made up of a network of fine fibrils embedded in an apparently structureless membrane The fibrils, after shadowing with uranium, showed a striated structure with a periodicity of about 650 Angstrom units On boiling, the fibrils, but not the underlying matrix, were dissolved. The fibrils were said to *differ from ordinary collagen in that no gel resulted on cooling* concentrated extracts

Ragan I think it is safest to leave it at the present time as an open question.

Porter I'll wager that they are ultimately very closely related The thing that should be emphasized is that the framework of all these fibers appears to be the same. They all have a basic 220 Å banding, at least morphologically, and anything else, like a 640 Å period, is superimposed on that.

Fremont-Smith: You might have it in a particular part of the kidney.

Porter. No, I doubt it very much There are, to be sure, great variations in collagens, variations in the width of the fiber and also in the degree of development of the 640 Å periodicity But all the extracellular fibers we have encountered have shown some evidence of striae

Angevine: Since you have said there are collagens, you will probably find this may be one of the collagens.

Fremont-Smith: Maybe these are reticulins.

Angevine. It would be better to stick to the larger one first

Meyer. By staining electron microscopy, classical methods, and in fact by every method, the fibers are chemically different from collagen. However, they say that full data have not yet been obtained.

Travell: We should say "different from the known collagens"

Porter: Yes, of course there is that difference, but I feel that we should emphasize the similarities and not the differences

Fremont-Smith: Dr. Porter, I don't quite understand your objection to what Dr. Meyer reported?

Porter. I have no right to object because I haven't seen the paper he is discussing

Holbrook: Dr Porter has seen 220 Å periodicity in all reticulum which he has examined.

Porter I am simply saying that the basic structure, or a variation on it, is descriptive of differences

Fremont-Smith: You worked with the kidney?

Porter. No, I haven't.

Fremont-Smith. Then it seems to me fair to say that you have no argument with this report on the kidney

Porter If I were acquainted with the paper I might find other reasons to doubt reasons arising from technique or interpretation In more and more areas, and more and more tissues of the body, we are finding similarities that permit generalizations. Some people don't recognize the 220 Å periodicity in collagen fibers

Fremont-Smith: These authors apparently do.

Ragan: At our Second Conference we heard from Dr Lansing about the physical structure of the elastic fibers. I haven't heard anything that makes me change my mind about that Have you, Dr Dempsey?

Dempsey: Dr. Tunbridge, when he was over here last year, rather disagreed with us His particular point was that in so-called

physiological states. There has not been, so far as I know, any study on turnover done in situations in which one might expect the rapid laying down of collagen, or more importantly, the rapid destruction of collagen. I don't think this is a closed issue. This is a place, Dr Fremont-Smith, where I think premature agreement, on the basis of our present data, would be too bad, I think more research is needed.

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Porter: I'll wager that they are ultimately very closely related The thing that should be emphasized is that the framework of all these fibers appears to be the same. They all have a basic 220 Å banding, at least morphologically, and anything else, like a 640 Å period, is superimposed on that.

Fremont-Smith: You might have it in a particular part of the kidney.

Porter. No, I doubt it very much. There are, to be sure, great variations in collagens, variations in the width of the fiber and also in the degree of development of the 640 Å periodicity But all the extracellular fibers we have encountered have shown some evidence of striae.

Angevine: Since you have said there are collagens, you will probably find this may be one of the collagens.

Fremont-Smith. Maybe these are reticulins

Angevine. It would be better to stick to the larger one first.

Meyer: By staining electron microscopy, classical methods, and in fact by every method, the fibers are chemically different from collagen However, they say that full data have not yet been obtained

Travell We should say "different from the known collagens"

Porter. Yes, of course there is that difference, but I feel that we should emphasize the similarities and not the differences

Fremont-Smith: Dr Porter, I don't quite understand your objection to what Dr. Meyer reported?

Porter. I have no right to object because I haven't seen the paper he is discussing

Holbrook. Dr. Porter has seen 220 Å periodicity in all reticulum which he has examined

Porter. I am simply saying that the basic structure, or a variation on it, is descriptive of differences

Fremont-Smith· You worked with the kidney?

Porter. No, I haven't

Fremont-Smith. Then it seems to me fair to say that you have no argument with this report on the kidney.

Porter· If I were acquainted with the paper I might find other reasons to doubt reasons arising from technique or interpretation In more and more areas, and more and more tissues of the body, we are finding similarities that permit generalizations Some people don't recognize the 220 Å periodicity in collagen fibers

Fremont-Smith: These authors apparently do

Ragan At our Second Conference we heard from Dr Lansing about the physical structure of the elastic fibers I haven't heard anything that makes me change my mind about that Have you, Dr Dempsey?

Dempsey: Dr Tunbridge, when he was over here last year, rather disagreed with us His particular point was that in so-called

elastosis, a pathological modification of elastic tissue in the skin, the material which stains like elastic tissue is not elastin at all It is not even modified elastin but is, indeed, a modified collagen. Carrying this to its conclusion, if a collagen may be so modified that it gives all the physical reactions of elastin, then there is no such thing as elastin.

Ragan: Was this based entirely on the histological picture?

Dempsey: On electron microscopy and conception, Tunbridge says that if one examines an area of elastosis, one doesn't see anything in it but collagen, which, incidentally, is a factual point with which we disagree.

Ragan. We can say, then, there is some difference of opinion, which is not completely resolved.

Holbrook: The difference of opinion would be largely in the derivation, Dr Ragan.

Ragan· No, in the physical structure, and in the chemical composition I don't think there is any knowledge about derivation.

Holbrook· Except that Tunbridge says it is a form of collagen.

Meyer: Is that true only for this disease, elastosis?

Dempsey: Yes, but if in this disease, or in any situation, you can produce a collagen which gives all the reactions of elastin, then where are your definitions for elastin? There is no such thing

Meyer. I am not sure whether I know a definition for elastin except that it is chemically and physically different from collagen I do not believe that there is any disagreement about the difference between collagen and elastin.

Dempsey· There isn't. But, on the other hand, there hasn't been an opportunity for disagreement because I believe the only recent papers on composition are those which have come from our laboratory, and nobody has yet denied them.

Ragan: That is what I meant by saying there was unanimity of opinion, because nobody else has done anything on it.

Meyer As far as I remember, in tissue culture elastin fibers are only produced from transplants of whatever the cells are from regions in which, in the adult, elastic tissue appears, such as from the region of the *ligamentum nuchae*, or from the aorta.

Dempsey. I'm sorry that I interjected this issue I think it is perfectly fair to say there is general agreement that the physical characteristics, and chemical composition, of elastin are different from those of the other connective tissue fibers. Also, as to the cells that are in the neighborhood of forming elastin, I wouldn't say that

I could differentiate between fibroblasts or macrophages. I wouldn't know what to call them so the question is open.

Ragan: Is there any knowledge about the turnover of elastic tissue? I don't know of any.

Dempsey: None I don't think it has ever been studied.

Ragan: The only concept concerning changes with aging and trauma was that described by Lansing, and has that been denied?

Dempsey: No, there is a fair confirmation of most of it.

Ragan. So I think we could leave that item fairly clear-cut The next item, the interfibrillar material is where we really get mixed up I put "ground substance" in quotes

Meyer Substances

Ragan. Is that agreeable to everybody, to call it "ground substance"?

Travell: Dr Meyer suggested the plural.

Angevine. You say "protoplasms" I don't see why you couldn't say "substance" Don't you have many things in the substance? Why not just call it "ground substance"? Let's not complicate it

Travell. "Substance" being collective

Angevine Yes, like protoplasm

Fremont-Smith You are willing to have it accepted, though? You are willing to have it in quotes?

Angevine: Yes, but leave it in the singular

Ragan. On derivation, the only reports this conference group has had have been from Gersh, at the Second Conference in 1951 Gersh believes the fibroblast produces this material. You can add Asboe-Hansen's ideas about the mast cell You can add a lot of people's ideas, but they don't know. I put Gersh down here because he is the only one who has made a statement at one of these conferences I really think you should say "open at the present time." Now, under "mucopolysaccharides," I should have started in by saying "mucoproteins," or "mucoids"

Meyer. Presumably the term mucoproteins is better

Ragan And then break it down into protein component and polysaccharide component. Is it agreeable to everybody to say that the proteins are different from collagen in the sense that they have aromatic amino acids in the mucoprotein of the ground substance?

Meyer This problem has not been sufficiently studied For example, if you include cartilage, I do not know of any studies of the composition and nature of the proteins If there is a mucoprotein of chondroitin sulfate, has the protein, or proteins, aromatic amino acids?

Ragan: Tendon has; it is the only one that has been studied.

Meyer. The mucoproteins of tendon and heart valves, yes.

Fremont-Smith. Let us add in quotation marks, "Only these tissues have been studied."

Meyer: Yes I don't know whether there is agreement on that.

Ragan· There, again, only one person – Dr. Meyer – has done this work and it has never been confirmed or denied. Then one must discuss distribution of these polysaccharides and their chemical composition, and I think that is as far as we can go We can say that by isolation certain materials have been derived grossly from certain tissues As far as localization goes, most of the histochemical studies are somewhat behind chemical isolation. They have depended in great part upon staining techniques which are nonspecific They certainly do not differentiate between the various chondroitin sulfates. They depend upon enzymatic reactions with something like testicular hyaluronidase, which are, again, nonspecific. You see something like this in Asboe-Hansen's work, in which he talks about hyaluronic acid just because it is metachromatic and disappears after testicular hyaluronidase incubation. But I think not only Dr. Meyer, but Dr. Blix also has done work which confirms this, isn't it so?

Meyer Yes, there has been some

Ragan. Has anybody done anything about these various chondroitin sulfates except you, Dr Meyer?

Meyer: Apparently not. We now have isolated from cornea a new type of sulfated mucopolysaccharide composed of equimolar concentrations of N-acetylglucosamine, galactose and sulfate. It occurs there next to a chondroitin sulfate, and a sulfate-free hyaluronic-acid-like-polysaccharide I presume the new polysaccharide would show metachromasy, but how it behaves histologically, I do not know.

Ragan Detailed knowledge about localization is impossible We have some detailed knowledge about chemical composition *en masse*, but that doesn't localize the material. Does anybody want to add anything more about mucopolysaccharides? The proteins have not actually been identified, except Dr. Meyer's finding that they contain aromatic amino acids The last point, whether you have a mucoid or a mucoprotein; that is, whether the linkage between protein and the polysaccharide is a tight one or is loose, is a point upon which we could argue for a long time.

Meyer: I don't know whether I understand you correctly. There are two different problems about mucopolysaccharide complexes in

connective tissue. The one type is what we should like to call mucoproteins, in which acid mucopolysaccharides are more or less loosely bound to proteins, and presumably specific proteins, by polar bonds and/or van der Waals' forces. The other are the true mucoids, as, for example, the α_1 serum mucoid of Winzler and of Schmid, which contain carbohydrate and protein in covalent union The Boston group believes that such mucoids occur in connective tissue, I do not believe that this has been demonstrated

Angevine: Dr. Meyer, one thing came up in connection with this. Just before I left Madison, Dr. Deiss told me, and I believe he has discussed this with you, that he has isolated hyaluronic acid from heart valves and the aorta. I asked him his explanation for finding it in view of your negative findings. Apparently there was a difference in the extraction method, which he didn't think should make any difference He used sodium hydroxide for extraction, whereas you did phenol extraction Would that affect it?

Meyer. No, we did both

Angevine I don't believe Wisconsin cattle are superior to New York cattle, but he told me he went to the slaughterhouse himself, removed the tissues immediately and transported them to the laboratory at once I wondered if that could have been the differentiating factor, because you don't slaughter in Manhattan, do you?

Ragan Oh, yes, we do

Meyer: I do not know the age of the hearts I used, but they were stored sometimes for quite a long time It is possible that would make a difference

Angevine· We have found from our histological studies that if tissues have been in the icebox for 24 hours or more, the metachromatic material diminishes rapidly. Tissues must be fresh Frozen material is the most satisfactory When tissues are preserved in fixative for any length of time before embedding, much acid mucopolysaccharide diffuses out of the tissues

Meyer We found some hyaluronic acid in one out of four or five preparations of tendon. We have repeated the experiment, and have again found a significant, but not a large, amount

Angevine I suspect the amount must change from time to time under different conditions

Meyer: That was in pig hearts – the only tissue we took

Angevine Deiss worked on beef hearts He has since studied the human heart, and I believe found it there

Meyer: I do not know whether there are differences in species, but they may exist.

Ragan: Do you think we should add the statement that these relationships may change?

Angevine: I suspect that they may. Whether or not the methods improve, there is the possibility that some of these things we are now accepting as basic may well be altered. This has bothered us, because on the basis of histochemical studies we postulated that there probably was hyaluronic acid in the heart valve, and since there was supposed to be none, according to Dr. Meyer, we were somewhat worried.

Meyer I am working up a new batch right now. But there, again, I don't know how long the hearts had been in the icebox.

Ragan: They hadn't been in the icebox, they had been at −70° C.

Angevine: Frozen?

Ragan: Yes, in a deep freeze

Meyer: And then they were thawed?

Angevine: How long between removal from the animal and being placed in the deep freeze? That, to me, is the thing that would determine it

Ragan. For the time it takes to ride in a taxi from 40th to 168th Streets

Angevine. That's not too long

Ragan: It might help to insert a statement at this point that the relationships that have been found might change under certain conditions. We don't know. We then come to the abnormal states. Here we have marked disagreement We had no unanimity of opinion about what fibrinoid was, even after the paper was given by Dr Bennett at our First Conference

Bennett: That is correct, I led the discussion, but we know little about *fibrinoid*

Ragan. Is there any reason to change that opinion now?

Angevine: Let's not start that again.

Holbrook: No, we belabored that.

Fremont-Smith: Several people agreed with themselves.

Dempsey: Several even agreed with each other.

Ragan. In abnormal states, whether or not collagen degenerates is something about which there is not too much unanimity of opinion

Dempsey: Do you mean that there are people who actually believe a collagen molecule, once formed, never disappears in the body?

Ragan: Oh, no!

Angevine: You mean whether it fragments in the tissue itself?

Ragan. Yes. The time-honored phrase "collagen disease" is my particular concern, namely, whether in a collagen disease, the collagen degenerates.

Angevine. But I should like to think that any tissue could degenerate.

Travell That is an aging process

Angevine: Not necessarily

Fremont-Smith Your question is whether, in collagen disease, the collagen is diseased?

Ragan Yes The role of the ground substance in all these abnormal states is a matter for discussion, and that is as far as one can go

Next is the response to trauma. These are histologically pretty well delineated locally, but the exact role of each component is a bit obscure, and the exact reason for each step in the process is still more obscure Do you think that is fair?

Holbrook Yes, I think so

Ragan. How about some of the pathologists? Do you think it is fair, Dr Angevine, to state that the exact reason for the appearance of each component locally in a tissue, in response to trauma, is not completely understood?

Angevine. No, I don't think it is well established.

Ragan Do you agree, Dr Bennett?

Bennett Yes, I would agree

Ragan. Under "responses to trauma, systemically," I put antibodies This is oversimplification, I know Again, we have a great deal of factual knowledge, but

Fremont-Smith. Before you get to that, it seems to me that the phrase, "responses to trauma," needs a footnote It carries with it the implication that there is a common response to a stimulus It seems to me that we have gone beyond that now, and that there is no such thing. The organism's response to a stimulus depends upon the state of the organism and its past history, and not upon the stimulus You can get diametrically opposite responses to the same stimulus if you have the appropriate past history of the organism This is the general problem of a single causality against multiple causality It seems to me we ought to make it clear that we are not subscribing here to single causality Other things being equal (which means the history of the organism up to the time that you traumatized it), you will get a common response It is true that the only reason you get a common response to the same

stimulus from any organism or system is because their past history, pertinent to this exhibitional behavior, has been similar. With appropriately different past histories or states, they will respond differently to the same stimulus.

Holbrook If we accept that, Dr. Fremont-Smith, we must accept the corollary that you won't like, that many different types of stimuli can produce identical results from trauma if the organism is conditioned to make them So we get nowhere.

Fremont-Smith. I don't disagree with that. I think we are somewhere.

Holbrook. Likewise the same stimulus may produce a variety of responses.

Fremont-Smith. I quite agree with you, and I think it is very important that they be restated, because we fall into a very basic error when we assume that there is such a thing as a common response to a given stimulus We are constantly setting up experiments where we put the organism under stress, and we make the assumption that when we take fifteen guinea pigs and inject them all with pitressin, morphine, epinephrine, or anything else, that we are really measuring the response of the guinea pig to morphine. But sometimes we are only measuring the response to morphine of guinea pigs who have a particular history. If we set up appropriate conditions and the guinea pigs react in the opposite way—that is, if they stop breathing with morphine—we can make them overventilate with morphine, if the conditions prior to injecting the morphine are known I am just stating this; I don't know the experiment in detail

Dempsey: There is a well known observation that in cats morphine causes extreme excitement, whereas in other animals, it does not

Travell· A slowing of the respiration with morphine, or an acceleration of respiration, may be produced in the dog.

Fremont-Smith. You can produce diuresis or inhibit diuresis with pitressin

Travell: Slowing of the heart, or acceleration of the heart, may be produced with epinephrine.

Angevine: On the other hand, if ose a guinea p t to a constant temperature, or if y ize it with c or some such thing, a fairly si e may be each time.

Fremont-Smith: Provided the hi been the

Ragan: If we take your attitude, Dr. Fremont-Smith, it would be very difficult to set up any standardized experiments.

Fremont-Smith. That is exactly the truth

Angevine: But you are just trying to point out the awareness that this difficulty exists and are not discouraging the setting up of the experiment.

Fremont-Smith. The reason why experiments which look as though they were standardized may be set up, is because nature conveniently provides us with an environment which is constant with respect to the pertinent issues for some particular bit of behavior Let's take a pair of identical twins, one of them living in Argentina and the other in Canada, who, at the age of 63, develop diabetes in January Somebody will say, "This is obviously a predetermined, inherited characteristic, so strong that the twins develop diabetes at the same time, even though their environment is entirely different" What I am saying is that that part of their environment which was pertinent to the exhibition of diabetes is not different Any scientist can set up an environment appropriate to the development of diabetes, or on the other hand, one which is inappropriate, so that one twin gets diabetes and the other one does not

Meyer But not, Dr Fremont-Smith, in a genetically determined type of diabetes

Fremont-Smith All right, how do you feel about that?

Meyer: I don't know

Fremont-Smith I don't mean to get into a long argument here, but there is no such thing as inheritance operating in the absence of environment Inheritance can be demonstrated only in terms of behavior, and behavior is always a response of the organism to environment, yet if you put the organism in a different environment, pertinent to that behavior, it is going to exhibit different behavior I don't think you can get away from that

Holbrook. You can say that heredity may limit the potential.

Fremont-Smith Absolutely

Meyer· In other words, we can make experiments at this time only with a given breed of animal, and not with some conceptually changed animal which does not exist

Fremont-Smith That's right But, for instance, if you want to work on rats, you get pedigreed rats, and not only that but you put them on a constant diet

Meyer· It is important that you take litter mates

Fremont-Smith: But you discover that it doesn't work because the rats all come down with chronic lung disease, and you find yourself studying the reaction of lung-diseased rats and not the reaction of normal rats.

Porter. Oh, you could use enough rats so that you would be studying rats.

Fremont-Smith: You could study a hundred rats in your experiment. But 98 of the 100 may have lung disease, so you are still studying lung-diseased rats

Porter: That would be unusual and a catastrophe.

Fremont-Smith: Yes, but it is such a common catastrophe that rats' lungs are not studied very often. For instance take the study of aging rats which was made by Clive McCay at Cornell University. It happened that the Foundation made a grant, after the project had been going for some years, to provide a pathologist, and he discovered that the whole study had to be done over again because nearly all the rats had bronchiectasis I am making a philosophical point here, which I think needs to be made again and again, even in a group of this sort. Our first reaction when we encounter such difficulties is to say, "No, that can't be so, because if it were so, it would make life too difficult for us."

Ragan: It is very discouraging!

Fremont-Smith: But it doesn't have to be so discouraging.

Travell: *That is what makes experiments* on human beings *so* exceedingly difficult.

Fremont-Smith: That is why pharmacology is in such difficulties.

Dempsey: May I be even more philosophical, Dr. Fremont-Smith. That comes down to the fundamental axiom of the scientist, that if every relevant detail is controlled, the cause-and-effect sequence must be invariable, and that when the same stimulus is provided, the same response will always occur.

Fremont-Smith: Your word "relevant" includes the history, doesn't it?

Dempsey: Certainly, and therefore whenever we do two experiments, and one comes out one way and the other another, we automatically conclude that we missed a relevant detail somewhere.

Fremont-Smith: That's right, it is very encouraging.

Dempsey: Perhaps so, if we can locate that detail.

Fremont-Smith. All truths are only relatively true within the frame of reference in which they have been studied. You wouldn't disagree with that?

Dempsey: No

Fremont-Smith: Because each one has come to this table with a different past history with respect to these particular problems, and that past history means that different methods have been employed I don't mean to cast any aspersions; it is a good thing that we do have different points of view.

Ragan The point Dr Fremont-Smith mentioned was brought out during the discussion of antibodies last year, when Dr Fischel (10) stated that there are good antibody responders, and bad antibody responders. for instance, in a colony of rabbits, which are not similar so far as their past history goes But I think it is fair to say that in the responses to trauma, as measured systemically by antibody production, we have data which have been confirmed in many instances

I put down originally, "as modified by adrenal hormones," but I changed it, after Dr Fremont-Smith's speech, to "as modified by various agents"

Fremont-Smith I think you have done an extraordinary job, Dr. Ragan, in outlining this discussion

Holbrook· I think it was excellent, and perhaps there have been more areas of agreement than we have realized

Ragan We agree, then, that the connective tissues may be divided into three components, and that there are areas of agreement about the physical characteristics, the chemical composition of collagens, and the physicochemical composition of elastin.

Holbrook That is a good deal

Ragan What do you think we should do now? Shall we make this into a table, or wait and see how the discussion goes? If it is worth while making up the table, I should like everyone to make suggestions regarding it

Holbrook I think it is worth while, for more reasons than one

Bennett I think there should be an outline of this evening's discussion in the transactions of the conference A reader of this conversation would have a rather bad time if he didn't have some sort of outline in front of him

Holbrook· That's right It is hoped also that during the course of the conference we may pick out some vulnerable points

Porter Yes. It is good for problem definition It would be rather interesting for the reader, particularly the graduate student, to see the variety of comments that a group of this sort would make on such an outline

Dempsey. I am puzzled about what purpose there would be in publishing this discussion, or what would it be used for? I do not

see what a codification of what we may agree on at this hour and minute would serve. I don't think we would necessarily want to use it for teaching, because it would then become a didactic statement of "This is the way it is, boys, and you must take it." I am not quite sure that it accomplishes a purpose in the definition of problems, which rests much more upon an understanding of the detailed experiments (our background in making up this outline) than it does on a generalization, does it not? Is this not at too abstract a level to say, "Well, I'm going to do an experiment on the orientation of the fibrillar materials in normal structure"?

Holbrook: We haven't got to that part of it yet. I think there are very few men who could read our discussion at this meeting and get anything whatever out of it, unless they had a little outline to look at I don't care who they are; I think they would have a very difficult time. If what has been said here is to be published, then it seems to me that if it is to be read intelligently, it must include a little outline. That is not to say that our assumptions would be true or final.

Travell. An outline for the discussion, then

Fremont-Smith: I think the discussion has been a worth-while exhibition of humility, and really does show, to some extent, the state of knowledge in these particular areas, at least as felt by a group of this sort.

Dempsey: I am puzzled about what we are going to do with it after it is published.

Fremont-Smith: I assume, and I may be quite wrong, that almost anybody interested in the topic of connective tissue diseases would be fascinated to read what this group of people have been saying in this meeting I think we have been very interested; otherwise, we wouldn't still be puzzling about it.

Travell: It is a review of the past three years and it is a take-off for the next two sessions, isn't it?

Angevine: We are not much further than we were three years ago.

Travell: Oh, I think we are a lot further.

Angevine: I wouldn't say "a lot." But it might be considered as a sort of stock-taking, which is desirable. I think we are all individually further ahead because we may have looked more carefully at the other person's point of view.

Fremont-Smith: We are further in the sense that we *know* we are not further — something we didn't know three years ago!

Angevine: Yes, that's what I mean

Holbrook: I think you will remember that we spent a fair part of the First Conference trying to decide, first of all, what the diseases of connective tissue were, and having wholly given that up, we decided to leave the diseases out of it entirely and talk about connective tissue alone. So we have made a little progress.

Ragan: The way the outline was set up, there is a half page on normal structure and four lines on abnormal

Dempsey I am still worrying We mention subjects on which we all agree, but what does our agreement mean? We may all agree at the moment, and change our minds fifteen minutes later.

Fremont-Smith: It should be made very clear that the agreement was of this moment only, and subject to change without notice. I think the things on which we all agree are very unimportant There are very few of them, and it may be a good thing to comment on that fact.

Angevine: Why don't we call that "relative unanimity" instead of "agreement"?

Fremont-Smith. That's fine: partial conceptions

Travell. Isn't the purpose of such an outline merely to provide a guide for the reader of the discussion? We don't have to state in it whether we agreed or not. They are just topics that came up for discussion, in this order and sequence

Fremont-Smith: It is an agenda for the discussion, that's all

EDITOR'S NOTE It seemed to serve no purpose to compile a table of agreements, reached after the discussion The original table is included at the beginning of the discussion in order to help the reader through the maze

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ISOLATION AND CHARACTERIZATION OF MAMMALIAN STRIATED MYOFIBRILS*†

CHARLES A. ASHLEY, ARMIN F SCHICK, ALBERT ARASIMAVICIUS, and GEORGE M. HASS

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Hass. I should like to be interrupted at any time I know that many things are to be discussed in this meeting, and if you do not interrupt me at the time the thought occurs to you, you doubtless never will However, I should like to present all the material which I have prepared, and I welcome criticism and suggestions from you.

The work which I shall report has not been done by myself alone It has been shared by several people working in my laboratory, and in other laboratories as well, and there will be points with which I am not familiar We are not physiologists or chemists, but doctors and pathologists who have been working on a problem which, to many of you, will doubtless appear to be beyond our powers of solution I wish that Dr. Ashley, Dr. Schick, Mr Arasimavicius, and Mr Philpott were here. However, Dr Keith Porter is present, and I shall ask him to discuss certain aspects of the study

In the beginning, we thought that we might approach the problem of cardiac failure by isolating the contractile unit of the cardiac muscle cell We believed that, once having done so, we might be able to investigate it *in vitro* and gain a better insight into the mechanism of the intact muscular machinery About five or six years ago, Dr Armin Schick, who is by training a clinician, came back from the war and wished to work in the laboratory for a year or two for purposes of reorientation before again entering medical practice I asked him what he was interested in, and he said, "the

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S A Sprague Memorial Institute, a [illegible]

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Illinois College of Medicine

heart." That was the beginning He started on the problem of attempting to isolate the contractile unit of the cardiac muscle cell. He succeeded in isolating the myofibrils (1,2). His original procedure involved only a few essential steps: Fresh muscle was frozen and cut into thin sections with a cold microtome. The sections were transferred to phosphate buffer solution, pH 7, ionic strength 0.25, at 0° C. Commercial trypsin was added, and allowed to act for 30 to 45 minutes on the sections at that temperature. The trypsin seemed to act principally upon materials which bind the myofibrils together, for when tissues so treated were lightly compressed on a glass slide under a cover slip, the myofibrils separated from one another, *spilling out of the muscle cells. Figure 1 represents a frozen*

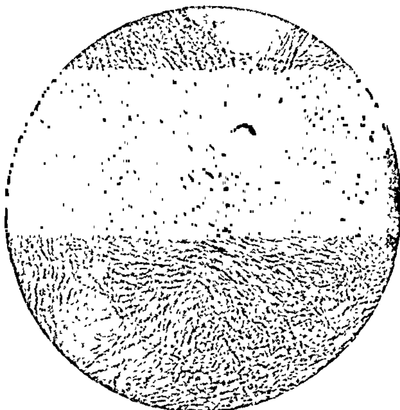


FIGURE 1 Low-power photomicrograph (x 450) showing the earliest stage of release of myofibrils from a thin section of cardiac muscle cells The section which has been treated with trypsin was lightly compressed under a cover slip prior to photomicrography

section of fresh muscle which has been squeezed under a cover slip on a glass slide and viewed directly with the light microscope. It is to be noted here that there is a disintegration of cell structure with a spilling out of the fine, delicate fibrils from the interior of the muscle cells.



Figure 2 illustrates the same effect. It shows skeletal muscle, which has been stained with phosphotungstic acid – hematoxylin to bring out more clearly the cross-band structure of the myofibrils during the process of mechanically induced disintegration of the muscle cell after treatment with trypsin

heart." That was the beginning. He started on the problem of attempting to isolate the contractile unit of the cardiac muscle cell. He succeeded in isolating the myofibrils (1,2). His original procedure involved only a few essential steps: Fresh muscle was frozen and cut into thin sections with a cold microtome. The sections were transferred to phosphate buffer solution, pH 7, ionic strength 0.25, at 0° C Commercial trypsin was added, and allowed to act for 30 to 45 minutes on the sections at that temperature. The trypsin seemed to act principally upon materials which bind the myofibrils together, for when tissues so treated were lightly compressed on a glass slide under a cover slip, the myofibrils separated from one another, spilling out of the muscle cells. Figure 1 represents a frozen

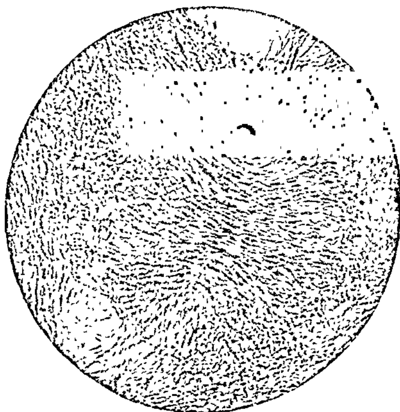


FIGURE 1 Low-power photomicrograph (x 450) showing the earliest stage of release of myofibrils from a thin section of cardiac muscle cells. The section which has been treated with trypsin was lightly compressed under a cover slip prior to photomicrography

section of fresh muscle which has been squeezed under a cover slip on a glass slide and viewed directly with the light microscope. It is to be noted here that there is a disintegration of cell structure with a spilling out of the fine, delicate fibrils from the interior of the muscle cells.



purification of mammalian striated myofibrils. Science [illegible] permission, from Schick, A. F., and Hass, G. M. The properties of mammalian striated myofibrils isolated by an enzymatic method. J. Exper. Med. 91, 655 (1950).

Figure 2 illustrates the same effect. It shows skeletal muscle, which has been stained with phosphotungstic acid—hematoxylin to bring out more clearly the cross-band structure of the myofibrils during the process of mechanically induced disintegration of the muscle cell after treatment with trypsin

Meyer Commercial trypsin — not crystalline — was used?

Hass: Yes. The same effect is obtained with crystalline trypsin. At this point, Dr Meyer, I should like to ask you what the action of trypsin, kept at 0° C. for a period of 30 to 45 minutes, is on protein, or a system such as we have dealt with here?

Meyer: I really don't know and do not remember any enzyme studies at 0° C

Porter. Might I ask what happens if you don't use trypsin?

Hass: With cardiac muscle, the myofibrils do not separate properly unless trypsin is used.

Fremont-Smith: It doesn't split up?

Hass: No; not with cardiac muscle, anyway. The myofibrils break up, rather than separate as intact long structures.

Holbrook: Do you have the impression, Dr. Hass, that the temperature may make the trypsin selective, that is, by changing the temperatures at which it acts, may it be selective at zero of the very binding fibers that you are trying to reach?

Hass: That is a possibility, Dr. Holbrook, as will be brought out later on. However, I am in agreement with Dr. Meyer about this, because when Dr. Schick came to me and told me that he was using trypsin at 0° C, I was surprised. He did it on his own initiative

Fremont-Smith: Did he say why he did it?

Hass: Yes, he said that he had worked with trypsin at higher temperatures, which I had advised, and had noted that the myofibrils separated from the cells, but that the myofibril structure disintegrated too quickly for him to exercise proper separation methods after they had spilled out of the muscle cell. So he used his own good sense and reduced the system to low temperatures. Around zero degree, he found that by limiting the time of action to 30-45 minutes he was able to induce this effect, which he viewed microscopically. He then proceeded with the isolation without undue disintegration of structure of myofibrils

Fremont-Smith· Then Dr. Holbrook's suggestion of a differential action of trypsin, as you lower the temperature, seems to be exactly the point

Hass. It may be, but I think that needs further investigation We have not solved the problem, nor have we spent much time trying to solve it, although I think it has interesting possibilities. I say this also for another reason. The commercial trypsin which Dr Schick used originally was a mixture of bacteria of every imaginable kind. The crystalline trypsin furnished by a manufac-

turer was also contaminated with bacteria, of which we isolated three types We found, when we attempted to convert crystalline trypsin into a bacteria-free preparation, that the activity of the product was greatly diminished I was surprised, and wrote to Dr Northrop, asking him how to sterilize crystalline trypsin. I do not recall the exact words of his reply, but I gathered that no special procedure had been used for sterilizing crystalline trypsin He suggested that we coat a Seitz filter with protein – I believe he said plasma proteins – and then filter a solution of crystalline trypsin through the coated filter We did this, and obtained so far as we know, a bacteria-free preparation, but the activity of the trypsin was greatly diminished Therefore, to those of you who are working with crystalline trypsin, I suggest that you study the bacterial composition of the preparation before drawing final conclusions with respect to what it does and does not do.

Holbrook. Do you mind telling us what organisms you found in the crystalline trypsin, if you remember? Were they always the same?

Hass No, they were not

Holbrook They differed, and had varying flora?

Hass: I do not recall the details, but at least three organisms were found, one was a small gram-positive coccus, another, a gram-negative bacillus, the nature of which was never defined, and the third was an anaerobic spore-forming microorganism

Holbrook. Are you intimating that some of the action which was assumed to follow the use of trypsin may well have been bacterial?

Hass Exactly I think it is important to labor the point because it has been a custom of biochemists, by and large, to work with preparations which they call enzymes, and many of them, I am sure, are hardly sterile during the preparation or the testing of their actions Aseptic techniques for preparation of enzymes, and the determination of enzymatic activity, should be a rigid requirement in any laboratory

Fremont-Smith This is compartmentalism, you see, where a chemist is working without a bacteriologist, and without a bacteriologist's slant on what an incubator does

Hass Yes, that's right

Fremont-Smith It is a nonbacteriological viewpoint where it is needed

Hass. The amounts of trypsin were rather small per mg of tissue: about 1/100th of a milligram, per mg of tissue, was all that was required

Fremont-Smith: Is that small, enzymatically speaking?

Hass. Yes, in many instances

Porter· The problem involved here, Dr. Hass, is one of digesting the cytoplasm between the myofibrils and thus liberating the fibrils. Now, might that not be just a solation of the cytoplasm, rather than a digestion of it?

Meyer. Yes, if you mean that it is a purely physical effect? One usually determines the temperature coefficient of an enzymatic reaction starting at about 10° C. and going up by 10° C. Perhaps it is wrong to extrapolate to 0° C. Day, in Leeds, published some work on the effect of trypsin on the ground substances of connective tissues and showed their very rapid removal. Unfortunately, he does not say how much trypsin was used in the experiments (3).

Hass: The principal effect here, as illustrated in Figures 1 and 2, was necessary in proper isolation of myofibrils from cardiac muscle cells, but was not essential for isolation of myofibrils from skeletal muscle Dr Schick's original procedure involved a thorough washing of the frozen sections after treatment with trypsin, and a reduction of the hydrogen ion concentration to 6.4, which is below the optimum for tryptic action. This was followed by a very brief treatment of the sections in a Waring blendor. It required only a few seconds in the blendor for the myofibrils to be separated from the structure in general and suspended in the medium The suspension was not a pure myofibril suspension: it consisted of nuclear fragments, whole nuclei, single myofibrils, and multiple myofibrils, in conjunction with one another. By differential centrifugation, at speeds varying from 400 to about 2000 rpm (revolutions per minute), a fairly pure preparation of myofibrils was obtained.

Travell This was from skeletal muscle, not from cardiac?

Hass. The same method was used for skeletal and cardiac muscle in the early studies. These preparations, viewed with the microscope, are shown in Figure 3 You will note that the myofibrils are well separated from one another They have not been stained and have been photographed in suspension; photographing unstained materials is difficult with ordinary equipment. It is to be noted that there is a change in the myofibril structure: the A disks are conspicuous, a little nodose and ovoid, the I disks are narrow. Furthermore it was clear, from staining methods and direct inspection, that the Z bands were almost always absent from these preparations.

Fremont-Smith: Which is the Z band?

Hass: The Z band is so small that it cannot be seen at this magnification I shall illustrate the bands later, Dr. Fremont-Smith

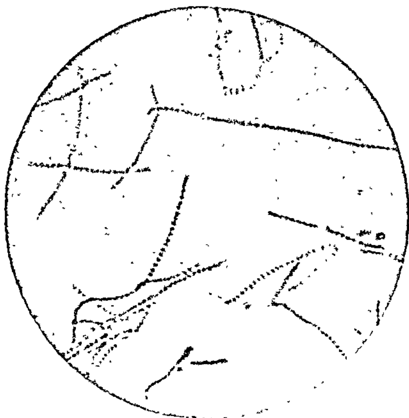


FIGURE 3 Photomicrograph (x 750) showing cardiac myofibrils of the rabbit in an early stage of purification

Figure 4 illustrates another preparation in which the dark A disks are elongated, and the intervening I disks are quite narrow Normally, the A and the I disks should be about the same length, so that there appears to be a change in the I disk at this magnification This is due to an effect of the preparative methods upon the structure of the I disk

This preliminary method was useful for purposes of demonstrating that myofibrils could be isolated in reasonably intact form, but for general purposes it had a number of limitations In the first place, the preparation of thin frozen sections of a large quantity of muscle was time-consuming and laborious It was also a cold procedure, for the preparations were made in the cold room at a

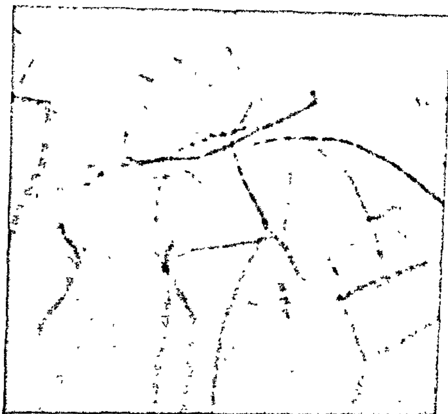


FIGURE 4 Photomicrograph (x 750) showing skeletal myofibrils of the rabbit in a late stage of purification Reprinted, by permission, from Schick, A F, and Hass, G M A new method for isolation and purification of mammalian striated myofibrils *Science* 109, *486 (1949)*, also by permission, from Schick, A F., and Hass, G M The properties of mammalian striated myofibrils isolated by an enzymatic method *J Exper Med* 91, *655 (1950)*.

temperature near 0° C A second difficulty with the procedure was in the control of the action of trypsin There was difficulty in obtaining identical action by trypsin in successive preparations Furthermore, as I have pointed out, the isolated myofibrils had certain deficiencies: the I disks were very narrow, and the Z bands were usually absent The use of other enzymes did not improve the method Papain had effects similar to, but less satisfactory than, those obtained with trypsin Pepsin was unsatisfactory.

For this reason, Dr. Ashley, Dr Schick and Mr. Arasimavicius went to work to develop more standardized methods for the production of myofibrils in large numbers (4). They found that skeletal muscle should be frozen at the time of death, and then stored

overnight in the cold room. When this muscle was transferred to
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of large yields, I mean hundreds of milligrams, where previously we had to work with only 15 to perhaps 50 mg. of myofibrils, dry weight.

It was also desirable to work out the conditions of substrate for proper isolation of intact structures. It was found that both potassium and phosphate ions were advantageous, and that sodium and chloride ions alone, as principal components of a substrate, were a deterrent to proper isolation Investigation was also made of the effects of ionic strength of the different buffer solutions used Ionic strengths between 0.154, and 0 25, were found suitable for isolation There seemed to be little difference in the yields of the isolation procedure in this range of ionic strength However, as the ionic strength of the solutions used for separation was increased above 0 25, the techniques failed, so that satisfactory isolations were not obtained when ionic strengths were above about 0.35 or 0 4 At 0 5, no success at all was realized (2)

The hydrogen ion concentration, most suitable for isolation, was also investigated, because it was our desire to find the ranges within which we could work and still preserve the structural integrity of the myofibrils. It was found that buffered media, pH 6 1-7 4, could be used in the isolation procedures, but that at pH 7 4-8, myofibrils developed a blurred structure

Meyer. Does all that you have said apply to these salts, plus enzyme?

Hass No, the data are concerned with the buffer media used in the final separation of myofibrils.

Meyer After the enzyme action?

Hass. Yes, after enzymatic action the enzyme was washed away from the preparation by multiple washing procedures and centrifugation Then, the myofibrils were resuspended in an enzyme-free substrate which was varied in composition, as just described. The isolation of the myofibrils *per se* was of considerable interest to us, but a matter of greater interest was the fact that when the isolated myofibrils were suspended in a buffer solution to which adenosinetriphosphate had been added, contraction of the myofibrils occurred

Porter Dr Hass, before you go into that, did you try any pH's below 6 1?

Hass: Yes, we did, but the present comments refer to the isolation procedures. When myofibrils are treated below a pH of about 5.1, the contractile component of the system is inactivated. I shall present data in a moment on the relations between solubility, contractility, and structural integrity of the isolated myofibrils. Dr. Ashley is conducting studies at the present time, in an effort to bring out the periodicity of structure below pH 5.

Fremont-Smith: In what degree was your ionic concentration different from that used by Dr. Baird Hastings, which was equivalent to intracellular ionic concentrations? I think he had magnesium ions, also, didn't he?

Hass: I don't know what the intracellular ionic strength is, but the physiologic ionic strength, so-called, is about 0.154.

Fremont-Smith: I mean, did you have any other ions besides potassium?

Hass. Not as principal cations in the buffer media, as I discussed them a few minutes ago. We used several other ions.

Fremont-Smith: That is another point. Dr. Hastings has that pretty well detailed. It is just possible that the additional ions that he used, which made it possible to obtain the approximate intracellular ionic concentration, might be helpful.

adenosinetriphosphate, they were elongated structures of the type shown in Figures 3 and 4.

Fremont-Smith: This shows contraction?

Hass: The final stage of contraction; it is one of the most interesting reactions that I have ever seen. I did not expect this to occur but Dr. Schick showed it to me one day. He used, in illustrating it to me, a barium salt of adenosinetriphosphate in the form of a commercial preparation. It contained other adenine nucleotides, but the principal component was the barium salt of adenosinetriphosphate.

Fremont-Smith: Is is an instantaneous affair?

Hass: It is so prompt that we have not been able to divide it, so to speak. We added a drop of a dilute solution of the barium salt of adenosinetriphosphate to myofibrils on a glass slide under a cover slip. Under direct microscopic vision we observed in sequence: first, the elongated structure of myofibrils; then they began to broaden; then the band structure disappeared; and finally the

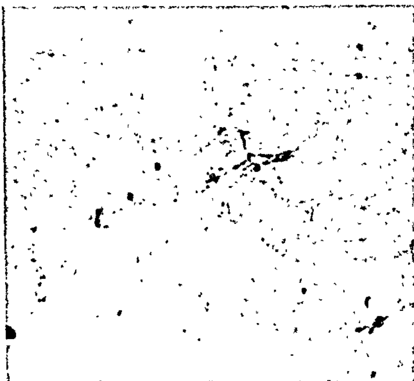


FIGURE 5 [illegible]

[illegible] *J Exper Med* 91, 655

myofibrils contracted into spherical, irregular, homogeneous masses, usually in a second or two

Angevine. Do they relax again, Dr. Hass?

Hass To our knowledge these fully contracted structures have never relaxed, nor have we been able to restore them to their original elongated form

Angevine Would it be more proper to term it syneresis rather than contraction, in view of something I may say later on?

Hass I think it is entirely proper to call this response anything you choose It is a response of the isolated myofibril, under certain

conditions, to the barium salt of adenosinetriphosphate. It is a form of response which we have not been able to duplicate by the addition of any chemical reagent, except *adenosinetriphosphate*. In the past year or two we have used the sodium salt of adenosinetriphosphate exclusively. The free acid is equally effective. The barium ion has no critical role.

Bennett: With reference to Figure 5, Dr Hass, and bringing it into relation to the others just shown, will you explain to us what you are demonstrating? What are these dark dots?

Hass: The dark dots are the end-result of the action of the barium salt of adenosinetriphosphate on the elongated structures shown in the previous Figures.

Fremont-Smith. On a single fibril?

Hass: Yes. Each one of the long single fibrils is converted into a tiny dot.

Fremont-Smith. Each one separately?

Hass· Yes, each fiber

Holbrook: Do you suspect they have lost mass and that this is only compression?

Fremont-Smith. Water has mass, hasn't it?

Hass. Yes.

Holbrook· In other words, these do not weigh as much as the fibril did before contracting?

Hass: I do not know.

Holbrook. I just wondered what your impression was.

Meyer. The Figure shows chains

Hass. Those are structures in the background of Figure 5.

Meyer: It is just not focused?

Hass· That's right, all are not in focus. It is a photograph of a deep preparation under a cover slip.

Meyer And if you used the sodium salt or potassium salt, would they have the same effect as adenosinetriphosphate?

Hass. Yes

Meyer. It is not the barium?

Hass: No, it is not the barium ion We have used the free acid and the potassium and sodium salts of ATP (adenosinetriphosphate), and the same effect was produced The barium ion was not necessary, and we have often removed it quantitatively from ATP preparations prior to their use in bringing about the contractile reaction of the myofibrils.

Bennett: The dark object in the center – the banded structure – is that related to the phenomenon of contraction?

Hass: I cannot recognize that as being anything except probably an agglutinated mass of structures They are not always as nicely separated as we should like to have them, they overlie one another, occur in little clumps, and are rather difficult to separate. But the contraction is a profound effect, without any question, and an effect which we have not been able to obtain with any reagent other than a reagent which is supposed to be adenosinetriphosphate.

The question concerning relaxation has been brought up by Dr. Angevine We believe that the degree of contraction, as shown here, is wholly unphysiological The contraction, as shown, is a total event that brings the myofibril to a state of disorganization, as will be demonstrated in the electron micrographs later on If we are ever to obtain relaxation, and I am quite sure that we shall be able to do so, it will be necessary to check the contractile event at a stage greatly proximal to the stage shown here. Physiologically, muscle does not ordinarily contract more than about 30 per cent of its length *When muscle contracts more, it is converted into an* unphysiological system – a delta state

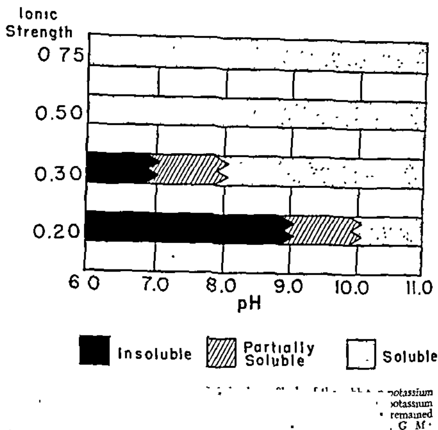
Porter As measured by what its inability to relax?

Hass Its inability to relax normally, and its inability, further, to contract in a proper fashion, to develop normal tension, and to do work normally. The illustrated degree of contraction, if you will accept that term, is far beyond that which occurs in the normal contraction of muscle The physiologist would be correct if he were to contend that we were dealing here with a totally unphysiological degree of contraction.

Fremont-Smith Worse than a cramp?

Hass· This contraction is worse than a tetanic contraction In general, one gets the impression that it is analagous to tetanic contraction of the myofibril Once this had been observed, it became clear to Dr. Schick that he had a problem to solve. The question arose as to how to study the system which had been isolated and made available for investigation. It was decided that for general purposes it would be advisable to determine the ranges of solubility of the isolated myofibril, and later to refer its stability characteristics in terms of form to those in terms of contractile function

Figure 6 illustrates the variation of solubility with pH and ionic strength of the media used for suspending the myofibril I wish to define the term "solubility," as used here These experiments were



... potassium
... potassium
... remained
... G M

The properties of mammalian striated myofibrils isolated by an enzymatic method *J Exper Med* 91, *655 (1950)*

done with small numbers of myofibrils placed under a cover slip and viewed directly with the microscope. Under direct vision, the isolated myofibrils presented themselves as beaded chains When *the beaded chain structure was no longer recognizable as such, we* had to say that it was soluble or that something else happened to it Therefore, if you will bear with me, the term "solubility" used here refers to an optical change, which makes the myofibril much less readily distinguishable, or indistinguishable when it is viewed with the microscope under a cover slip. It is to be noted that the myofibrils were not soluble at an ionic strength of 0 20 when the *pH range was from pH 6.0 to about pH 9 0. As the ionic strength* of the medium was increased to 0 50, the beaded structure of myofibrils entirely disappeared from the microscopic field in the pH range indicated in Figure 6

Meyer: Is this reversible or irreversible?

Hass: It is an irreversible effect.

Meyer: If you wash it with lower ionic-strength buffers, does it come back?

Hass: There is nothing in the Figure which has been shown to be reversible, except in the transition ranges.

Holbrook: It has been suggested that Dr. Hass orient some of us with regard to the myofibril and its parts so that we will know what he is talking about

Hass. All you have seen thus far in the figures are two alternating disks: the isotropic and the anisotropic disks The former is the one that has the least birefringence, and the latter is the one with the most birefringence. The isotropic, and the anisotropic, are the light and dark disks that you saw in the figures. A band goes across the middle of the A, or anisotropic disk, which is the M band, and adjacent to this is another band called the H band The I-A junction is where A and I disks join, and, as Dr. Porter will show you later these are longitudinal fibrous components which make up the bulk of the individual myofibrils Then, passing transversely through the middle of the I disk, there is the Z band which I pointed out as disappearing during the action of trypsin On each side of the Z band, several workers with the electron microscope have described other bands, J or N, and so on, but I think this should suffice for general orientation The A disks are in series Are there some things I left out, Dr Porter?

Porter There is the J or the I band?

Dempsey J is the same as I

Hass: These are the conspicuous bands, although there are probably a great many more yet undemonstrated

Dempsey May I say just one thing about that diagram something which Dr Hass mentioned but which I should like to underscore. The basic terminology here, "A," "I," "isotropic," and "anisotropic," are terms which refer to the appearance of these bands as seen with the polarizing microscope. It is also true that the A disk, when seen in ordinary light and with the ordinary microscope, is a dark band, so it can be recognized even without the polarizing microscope. It is also true that sometimes the A band will stain dark with certain stains, whereas with other stains it is the I band which is darkly colored Therefore, in straightening out the terminology, it is necessary to cross-check each preparation with the polarizing microscope in order to be sure that the bands have not been inverted That is of considerable importance, because it is

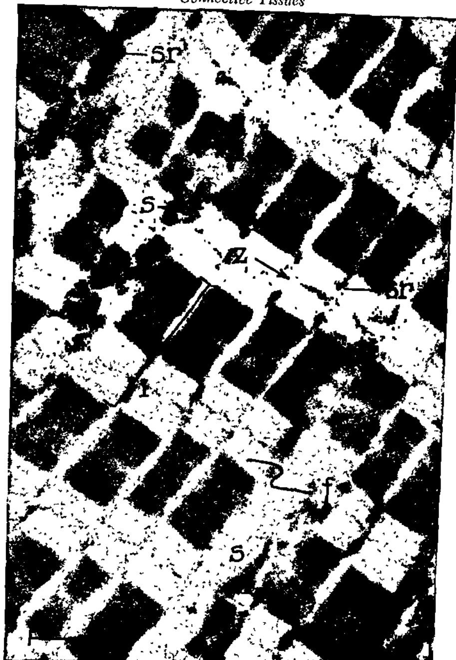


FIGURE 7 Longitudinal section of chick skeletal muscle Myofibrils (f) run diagonally from lower left to upper right The four in the center are essentially in phase The anisotropic, or A bands, are clearly more dense to the electron beam

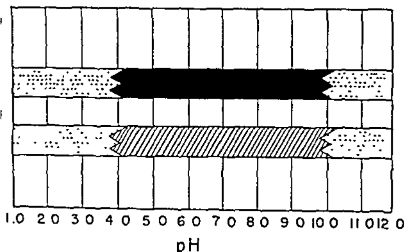
badly mixed up in many of the standard textbooks. Of the four standard textbooks in histology, for example, two of them have the A and the I bands reversed.

Porter. Dr. Hass, we all came prepared to help or hinder you and I think this is a good time to show Figure 7. The relaxed fiber in the electron microscope appears thus Would you point the bands out to us, Dr. Dempsey?

Dempsey: This section is stained with an eosin hematoxylin stain I think what Dr. Porter wants me to say is that the dark band is the A band, the light band is the I band, and, transecting the I, is the Z, and on either side of the I is an M band, which Dr. Hass has illustrated on the blackboard I have my own reservations about *this particular identification because the section is so thin; you can't* look at it with a polarizing microscope, and for some theoretical reasons that are neither here nor there, this could just be wrong.

Fremont-Smith: It is either that way or the other way.

Dempsey: Yes.



Hass: It was of interest to Dr. Schick to examine the relationship between the retention of birefringence by the use of polarizing light microscopy and hydrogen ion concentration in the medium I think Figure 8 will show the results in that regard.

This graph shows a close correspondence between the interpretation of *insolubility*, and the presence or absence of *birefringence*. This indicates, again, that we have not defined solubility in chemical terminology: our definition is strictly in terms of the appearance of the elongated myofibrils under the microscope. Many factors, of course, modify that appearance, but I assume that the most important modifications have come as the result of extraction of one or more important components from the structure of the myofibril. I have recently seen some preparations that Dr. John Ayer has made of elastic tissue. He began his study in my laboratory two or three years ago, and is now working with Dr. Warren in Boston It seems that the initial "solubility" in his preparations of elastic tissue means a continuous expansion of structure The transition to true solution is not clear, or at least it is mysterious.

Meyer It does not indicate simply a change in refractive index?

Hass I have no idea myself as to when some things are, or are not in solution

Fremont-Smith: Has anybody?

Hass. They may have. There are textbooks written on it! I am expressing the viewpoint of a pathologist but the viewpoint of a chemist is needed. This subject has been carried further in a correlation of the relationships between contractility, solubility (in quotation marks, if you will), ionic strength, and hydrogen ion concentration. A comparison was made between myofibrils isolated from human muscle and rabbit skeletal muscle. Figure 9 shows that there was no great difference in the basic properties of the myofibrils isolated from the two sources. There were minor differences at high ionic strengths, but these could be ascribed to differences in technical manipulation.

Angevine. Dr Hass, in view of something that may come up later, what muscle did you use from the rabbit? Was there any specific one?

Hass: We used the anterior thigh muscles

Angevine: Regularly?

Hass: Yes. The buffers contained potassium, phosphate and citric acid primarily. Replacement of the potassium ions by sodium ions made little difference in the results There is a difference between

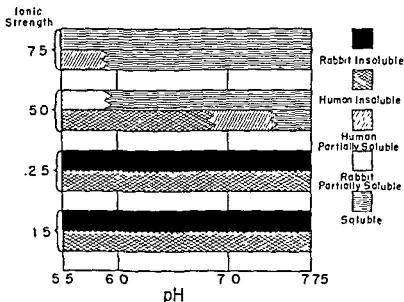


FIGURE 9 The comparative "solubilities" of human and rabbit skeletal myofibrils in buffer solutions with different hydrogen ion concentrations and ionic strengths

rabbit and human skeletal myofibrils after isolation, but we do not attach much significance to it. Perhaps, a difference in the dimensions of the myofibrils leads to these findings. The myofibrils isolated from human muscle are usually of greater dimensions than those isolated from rabbit muscle.

Fremont-Smith In all directions, both width and length?

Hass. That is a problem which will be discussed in a few moments.

Dempsey I'm sorry, Dr. Hass, but I don't understand the comparison that is made on Figure 9 The two bars go across at each ionic strength?

Hass: The black bar indicates the range of insolubility of rabbit myofibrils, and along with it, the cross-hatched, screenlike bar represents insolubility of human myofibrils On the abscissa, the hydrogen ion concentrations of the solutions used, from 5.5 to 7.75, are depicted, and on the ordinate, the ionic strengths of the solu-

ties throughout the range of pH used Also at an ionic strength of .25, the human and rabbit myofibrils have the same solubility

properties throughout the range of hydrogen ion concentration used At an ionic strength of .5, which is at limits of retention of structure of the myofibril, there is a little difference. As indicated in the diagram, the rabbit myofibrils are more soluble. At an ionic strength of .75 the myofibrils from both sources seemed to be soluble throughout the range of pH 5.5 to 7.75. These observations meant that we could work with human and rabbit myofibrils interchangeably and with the same substrates, without encountering difficulties from the standpoint of different solubilities

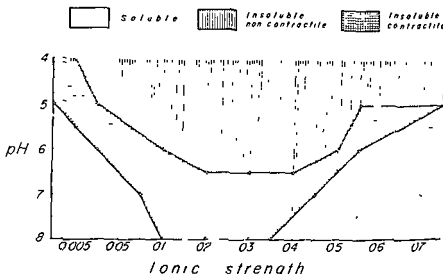


FIGURE 10 The ranges of "solubility" of skeletal myofibrils of the rabbit in potassium phosphate and citric acid buffer solutions of various ionic strength and pH The retention or loss of contractility on addition of 0 001 molar ATP is shown

Figure 10 is more complicated As Dr. Holbrook said, one should never put more than one item on a lantern slide, but there is a limit to one's capacity to carry lantern slides. Hence, it becomes necessary at times to include more than one observation. Figure 10 shows the solubility of skeletal myofibrils in potassium phosphate – citric acid buffers of different ionic strengths and pH values, and the limits of contractility on the addition of 1/1000th molar adenosinetriphosphate This is an interesting figure because it shows that the contractility properties of the myofibril persisted at physiological ranges of hydrogen ion concentration and ionic strength Contractility was retained between the levels of about pH 6 to

pH 8. At lower pH values there was a retention, within certain limits, of contractility in association with insolubility. This was particularly apparent at relatively high and low ionic strengths. The most interesting part of the figure, however, is the central portion. Here, there was a transition between the myofibril with apparent preservation of structure, which was contractile, and the myofibril which, though it retained perfect preservation of structure, was noncontractile. This transition range in the physiological range of ionic strength was somewhere between a pH of 5 0 and 6.2. Actually, it was closer to pH 5 in many experiments with different preparations

Dempsey Perhaps I am asking a question which you intend to discuss, but is it not interesting that the range is precisely the one which you found most effective in preparing your isolated myofibrils that is, between perhaps pH 6 5, and as high as 7 5, and with low ionic strength?

Hass That is correct

Dempsey: If one removed some protein by solution at those ionic strengths and pH, in the course of making the preparation, does this not suggest that physiologically the effect of that soluble protein may have maintained the contractility of that fibril?

Hass You are implying that the supernatant solutions in which we determine the contractile properties of the myofibrils contain proteins derived from the myofibril itself?

Dempsey Or perhaps surrounding the myofibril

Hass The myofibrils are very thoroughly washed The protein concentration in the supernatant fluids is so low that it requires Folin's protein reagent to define the amount quantitatively

Dempsey: But in life there is a high protein concentration surrounding them This protein certainly modifies the physicochemical nature of the interfibrillar milieu For example, it changes the dielectric relationships between the fibril and adjacent fibrils, and between adjacent protein molecules Thus, isn't it possible that in life, in the high protein milieu in which these things exist, that the contractility is maintained over a somewhat different pH and ionic strength range than you have here?

Hass I think that is entirely likely

Dempsey. I am wondering about the effect of what you removed. That is what I am trying to get at

Hass I think it is entirely likely, Dr Dempsey, that there are many modifying factors in intact muscle This is a wholly isolated preparation The medium contained little or no protein in the

supernatant. The less the amount of protein in the supernatant fluid, the happier we were in the analysis and the acceptance of the results. Protein is an ever present disturbing factor in almost any type of enzymatic or other analytical determination of biological systems.

Dempsey: Yes, but the protein is there, and when the thing works in life . . .

Hass: The protein is there in the intact cell We have not come to the point where we can interpret these results in terms of the living cell. I doubt whether it will ever be possible to do so But I wish to emphasize again that there is an interesting and rather wide range of hydrogen ion concentration and ionic strength where preservation of structure and contractility seems to be excellent. Once you transgress the particular limiting range you encounter a situation in which the myofibril becomes noncontractile, even though structure is retained Furthermore, the wider the divergence of ionic strength from the physiological range, the better the preservation of the contractile property at low levels of pH.

Porter: It is interesting that the range coincides with the probable range within the living cell. To that extent, we can extrapolate.

Hass: Yes, it points to studies which are being conducted at the present time These are concerned with what happens to the

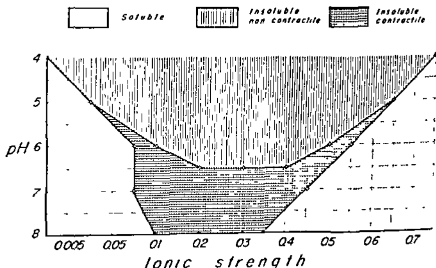


FIGURE 11. The "solubility" of cardiac myofibrils of the rabbit in potassium phosphate and citric acid buffer solutions The limits of retention of contractility of the myofibrils on addition of 0 001 molar ATP are shown.

myofibril to render it noncontractile, under conditions indicated in the diagram.

If you will fix Figure 10 in your minds for a moment, I shall show Figure 11, which represents the same type of investigation conducted on cardiac myofibrils under identical conditions. You will notice that there is a great similarity in the responses of isolated skeletal and cardiac myofibrils. It is almost too close to be true. Certainly, the errors involved in the technique might account for most of the divergence in the two figures

Dr. Ashley at this time became interested in the possibility of studying the fine structure of the fibrils which had been isolated. He felt certain that preparations could be made which would be suitable for electron microscopy He made the preparations and called for assistance in electron microscopic work and interpretation from Dr. Keith Porter, and from Mr. Delbert E Philpott, who was at that time at the University of Illinois (4) I should like to have Dr. Porter discuss this because he is an authority on this subject and these are, for the most part, micrographs which he made with Dr Ashley at the Rockefeller Institute.

Porter I cannot agree with you, Dr Hass You had better discuss them as you see them

Hass You mean that there will be a discussion if I don't see them the same way? It is very likely that we shall not interpret them in the same way; I am not familiar with this type of analysis These myofibrils were isolated by the colloid mill method, so that they show preservation of structure both in the A and I disks In Figure 12, are shown the A disk, the I disk, and the Z band. I think, if you look closely, you will see the M and H bands in the A disk The little tracks in the I disk may represent one of the N bands described by Hall, Jakus and Schmitt (5), or the collapse of the Z band

Travell. Hasn't the I disk shrunk?

Hass Yes, the I disks invariably show shrinkage in these preparations

Bennett This is a skeletal myofibril, isn't it?

Hass Yes The M band bisects the A disk, and on each side of the M band there is a little trough of decreased density Then there is more laterally an area of increased electron density, the H band, which lies on both sides of the M band The other part, which is shown here in the figure, is the so-called longitudinal fibrous component This is represented by parallel longitudinal striations, as indicated in the figure. The longitudinal fibrous component, accord-

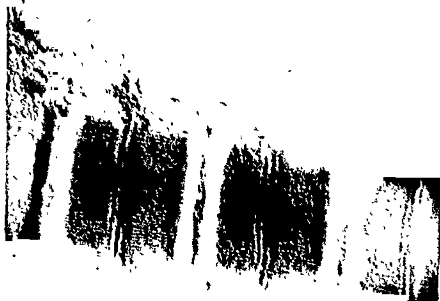


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ing to other workers, has aligned along it, or as part of it, a periodic nodosity, which is supposed to be at intervals of 400 Angstrom units Drs Porter and Ashley came to the conclusion that the nodosity along the fibrous components in their preparations was irregular and had no specific periodic spacing (4). Another point which they bring out in their paper on this subject is that there is no apparent continuity between a fibrous component, let us say, as it traverses the I disk, and a fibrous component which lies in the A disk. Other workers have shown that there is a continuity of the longitudinal fibrous component throughout the length of the myofibril.



FIGURE 13 Thin section of myofibrils from chick skeletal muscle was taken to show slender myofilaments which are organized in parallel array in A bands of fibrils. In favorable specimens these filaments can be seen to traverse I band as well as A band. They are spaced about 350 Å apart and show a faint longitudinal periodicity measuring 225 Å. The filaments themselves vary in width between 100 and 150 Å. (x 47,000.) Reprinted, by permission, from Bennett, H. S., and Porter, K. R. An electron microscope study of sectioned breast muscle of the domestic fowl. *Am. J. Anat.* 93, *61 (1953)*.

Porter: I am quite sure there is, Dr. Hass. This type of preparation doesn't allow you to see it, but others do.

Hass: In any event, the continuity is not shown here.

Porter. Might you not say that this is a partly contracted myofibril? The I band is definitely shortened, and even the thickening along the end may represent some contraction.

Hass: Yes. It is fair to say that all myofibrils to be shown in the electron micrographs have undergone some degree of contraction, because of the method of preparation. The mere fact that the muscle has been frozen and handled in the way described previously would indicate that some degree of contraction probably occurred during the isolation of myofibrils.

Porter: Is this an appropriate point at which to show the fine structure of myofibrils?

Hass. I should like you to do that for us.

Porter: Figure 13 can be easily interpreted It is a micrograph of a thin section, through a fiber such as Dr. Hass has just shown you. The long band in the center, the A band, is traversed by the myofilaments, which are evenly spaced and arranged parallel to one another. They are about 100 Å in diameter, and 300 Å from center to center The narrower, dense bands at the upper right, and the lower left, represent the Z bands, with the I around them on either side.

Fremont-Smith: Is there any periodicity?

Porter: There is some that can be detected, but it is not well defined in this preparation

Hass: Dr. Porter has indicated that the average breadth of these filaments is somewhere in the neighborhood of 100 to 200 Angstrom units, which is approximately four or five times the presumed molecular diameter of most proteins. Thus, this is not very far removed from a molecular diagram Is that correct, Dr. Porter?

Porter. That is right, and I think it is interesting for this group to see it in cross section while we are about it. The cross-sectional form of the myofibril is irregular in outline, but you can see the dots which represent cross sections of the filaments imbedded in the A substance, and when these are examined at high magnifications the organization is found to be highly ordered (Figure 14). The myofilaments are closely packed, you see, with the rows arranged at 60 degrees to one another.

Hass: This is the tiniest component that you resolve, and do you believe that the myofibril is tubular?



FIGURE 14 Cross section through chick skeletal muscle The myofibrils (xf) are irregular in outline and vary from dense to less dense as plane of section passes from A to I bands (as at x) They are surrounded by sarcoplasm (s) containing elements of the sarcoplasmic reticulum (sr) The dense dots in the myofibrils represent cross sections (end views) of myofilaments which are closely packed in hexagonal arrays (x 40 000) Reprinted, by permission, from Bennett, H S and Porter, K R An electron microscope study of sectioned breast muscle of the domestic fowl Am J Anat 93, 61 (1953)

Porter: I do not know. The myofilament you refer to may look tubular there, but I doubt it.

Hass: That was your contention in the beginning.

Porter. No, we contended along with some others that the myofibril, not the myofilament, is hollow.

Hass. Well, it is a beautiful micrograph.

Porter. I think it is a good time to point out, also, Dr. Hass, that the material around the fibrils is the sarcoplasm; it has some components which you may want me to refer to later, perhaps, in the discussion

Hass: Yes.

Fremont-Smith Which is the sarcoplasm here – the light part?

Porter: Yes, the light material between the fibrils

Dempsey: With globs of darker material over them



FIGURE 15 Electron micrograph (x 12,000) of a rabbit skeletal myofibril, isolated with a colloid mill, fixed with formalin and shadowed with chromium

Hass. That is the material we lose for the most part when we separate myofibrils from the other cytoplasmic components

Travell This is a section through the A disk?

Porter. Yes, in part.

Angevine. That probably is a mitochondrion at one o'clock?

Hass Could this be a mitochondrial structure?

Porter No, it isn't big enough, it is something else.

Hass When we prepare myofibrils the intervening material is lost in making the preparation The filaments and adherent materials are retained in the isolated myofibrils

Travell Where do you get the 60-degree angle you spoke of?

Porter It is seen in the arrangement of the filaments It describes the filaments as having uniform diameters, and associating with the material around them, which is referred to as the A substance

Fremont-Smith Hexagonal, like the eye of an insect?

Porter Yes, that's right.

Fremont-Smith Multiple eyes?

Porter: Yes. I'm sorry, Dr Hass, but I thought it was a good time to clear up the morphology

Hass That is a good illustration Figure 15 brings out the structure of the filaments An A disk has been crushed, or has partly disintegrated, so that the longitudinal filaments are sprayed out in the field These have been shadowed with chromium The filaments are rather straight rods with a little nodosity, which is presumably due to the absorption or the binding of so-called "A" substance on the elementary filament structure The figure shows the M band, the I disk, the A disk, and the Z band This is a colloid mill preparation of a skeletal myofibril, Dr Meyer, without use of trypsin.

I think Figure 16 is a particularly fine illustration It was the one which Drs Porter and Ashley debated about at considerable length There was a thesis which was proposed, I believe, by Draper and Hodge (6), or Pease and Baker (7), that the myofibril was more in the nature of a tubular structure, or perhaps a coiled ribbon The reason for this contention was that the myofibrils when they were dried out on the screen and shadowed, showed so little evidence of central axial elevation They seemed to be more or less planar in configuration rather than cylindrical This was interpreted by them, and others, as indicative of something else than a solid cylindrical structure In Figure 16 one of the A disks seemed to have been on end Dr Porter was of the opinion that this might



FIGURE 16 Electron micrograph (x 21,000) of myofibrils, isolated with trypsin, fixed with formalin and shadowed with chromium Reprinted, by permission, from Ashley, C A, Porter, K R, Philpott, D E, and Hass, G M. Observations by electron microscopy on contraction of skeletal myofibrils induced with adenosinetriphosphate. *J Exper Med* 94, 9 (1951)

represent a kind of an angle photograph down into the inside of an A disk. Is that correct, Dr. Porter?

Porter: Well, I am afraid in the light of later discoveries it is not. But that was the interpretation at the time, Dr. Hass

Hass. The interpretation was, in part, largely justifiable because after all one must admit that there is a great deal of planar configuration in these preparations. This would not necessarily be expected if the myofibril were a compact cylindrical arrangement of tiny elementary filaments or fibrous components If it is a solidly aligned structure, without any "vacuum" or hollow space in the center, certain lines of reasoning follow. If it is essentially tubular in character, with the elementary filaments aligned around a hollow

space in the center which contains other materials, the approach toward an explanation of the behavior of muscle would be slightly different.

Dr. Ashley felt that it might be possible to make a study of the isolated myofibrils in various stages of contraction (4) It was *clear that this study could not be made under conditions ordinarily* used in determining the contraction of isolated myofibrils after the addition of adenosinetriphosphate As a consequence, he made a buffer solution which contained glycerine and brought the myofibrils, after isolation, to a temperature of about −5° C. Under these conditions the myofibril did not contract on the addition of adenosinetriphosphate to a final concentration of 0 0025 molar, but *remained unchanged in a noncontractile state. The myofibrils* were then transferred in the glycerinated buffer solution at −5° C to a collodion-covered wire screen maintained in a cold room at 8° C As the temperature of the suspension of myofibrils on the screen rose from −5° to 8° C, contraction occurred. The contraction was slow, requiring about one minute for completion Myofibrils in successive stages of contraction were obtained by formalin fixation of a series of preparations at intervals of fifteen seconds After formalin fixation, the myofibrils were washed on the screens, dried, shadowed with chromium, and studied by *electron microscopy*

Figure 17 shows a skeletal myofibril It illustrates progressive stages of contraction along the length of a single myofibril One part has a nearly normal structure, although the I disks and Z bands are missing, as in most trypsin-isolated preparations The M band is quite distinct, and as you follow the contraction, which becomes of greater magnitude on passing to the other end of the fibril, it is noted that the breadth of the myofibril increases Also, the myofibril shortens, as shown by the decreased distance between successive M bands There appears to be a movement of material from the H bands towards the A-I junctions

Fremont-Smith. Please explain that I am not quite clear on it.

Hass. As you move from the left along the long axis of the myofibril, you move into a contractile phase One M band, and an adjacent band, may be seen There seems to be an accumulation of material which lies in the A-I band region, and not directly in the A disk It lies somewhere in the neighborhood of the junction of the A and I disks Why is this interpreted as migration of material? Because as material accumulates in this area, there is a *diminishing density of the intervening zone* As the degree of contraction increases there is an accumulation of more material in the



FIGURE 17 Electron micrograph (x 13,500) of a rabbit skeletal myofibril, isolated with trypsin, contracted with ATP at 0° to 8° C, fixed with formalin and shadowed with chromium

A-I-Z region, and further on there is an accumulation of still more material in the same position You can still see the faint residual trace of the M bands. Further along the M band disappears, and all other bands likewise disappear All that remains are the contraction bands, which so far as can be determined, represent new band structure which develops as the other bands disappear Only the filamentous longitudinal components remain as readily resolvable structures

Dempsey: May I interject something here? I think you pointed it our earlier that this type of contraction is probably something like the supercontracted state, or over and beyond the type of shortening that occurs physiologically. The reason is that in the normal light microscope histology of muscle, relaxed muscle has approximately a 50-50 ratio between the A and I bands In the

contracted muscle, most of the shortening occurs in the I band, so that in such a contracted muscle the A band appears disproportionately broad to the I band Now, in almost all these preparations, the I band seems to be short, so it looks as though you are starting out with a muscle that has already shortened to about its physiological limit, and there is something superimposed on top of that Would you agree with that interpretation?

Hass: It is possible that this is, perhaps, beyond the limit of physiological shortening of the muscle, but the isolated myofibril itself has capacities to shorten far beyond the so-called physiological limits of intact muscle

Dempsey: Yes, this is like the supercontraction that you illustrated in the isolated filaments, a little earlier.

Hass: Yes, this approximates the final supercontracted form.

Holbrook: Except that it is not completed

Hass: It is not total disorganization at this stage.

Holbrook: One more question, Dr Hass If you move it from the −5° room into the 8° room and catch the earliest phase that you can measure of contraction and take it back into the −5° room, is it still not reversible?

Hass. That, Dr Holbrook, is an important question In other words, our capacity to define the earliest stages of contraction is limited The capacity to define by this method advanced stages of contraction is, as you see, satisfactory The very earliest stage in contraction has not been demonstrated in these three preparations, unless you wish to accept the figures as illustrative of earliest evidences of contraction As I stated before, if we ever determine reversibility of the system, we are going to have to stop the system in an early stage of contraction and reverse it, I would say, after it has gone no further than something of the magnitude shown We have not succeeded in doing this up to the present time

Porter You would have to say that this represents a contraction of the A band rather than the whole sarcomere, since the I bands have disappeared

Hass The I disks are almost absent, and the Z bands are completely gone, due largely to the initial treatment with trypsin

Porter Doesn't it look to you as a sort of reaction that creeps up into the A band from the A-I junction?

Hass I am simply accepting original interpretations, Dr Porter

Porter That's risky!

Hass The interpretation originally made by Drs Ashley and Porter was that this represented primarily a migration of material



FIGURE 18 Electron micrograph (x 13,500) of rabbit skeletal myofibrils isolated with colloid mill, suspended in glycerinated buffer solution, contracted with ATP at 0° to 8° C, fixed with formalin and shadowed with chromium

from the H bands away from the M bands. In going over the electron micrographs, I could not personally specify exactly what was happening. I didn't understand where this material in the contraction bands was all coming from.

Figure 18, will illustrate it further. This is a colloid mill preparation Trypsin was not used, and the Z bands have been retained. This is a partly contracted preparation; the upper myofibril is in a more advanced stage of contraction. You can see the M bands in the lower myofibril, and somewhere in the elevations are the I disks, or what is left of the I disks, plus this accumulation of material which seems to be in the A-I-Z region.

Porter: You have to make the distinction here, though, that trypsin was not used, and the I bands were left in place

Hass: Yes, that's right.

Porter: In other words, this is more representative of the actual myofibril as it exists in the cell.

Meyer: But this was also done with the same technique, using glycerin and ATP?

Hass. Yes, all of these are adenosinetriphosphate-contracted.

Meyer: In glycerin solution?

Hass: Yes, a glycerinated buffer solution was used

Travell: This is skeletal muscle?

Hass Yes It was isolated without trypsin, but with a colloid mill

Porter: Dr Hass, there was no indication of the density of the A band before you initiated contraction

Hass. We have no indication of it.

Porter: So you cannot say how much of it has lost by way of this contraction?

Hass: No This gives you an idea, though, of the remarkable events that seem to be taking place The A disks are almost planar between contraction bands represented by marked elevations at the A-I junction, and apparently in the I band region You can still make out the M bands despite conspicuous contraction bands

Angevine. Have you any observations, Dr Hass, on what has been happening to the adenosinetriphosphate in the myofibril as these changes are taking place?

Hass. That will be discussed later. Figure 19 illustrates the same effect The coordination here of a rather well-contracted myofibril against a myofibril which is less contracted indicates spatially, again, the relationships which exist In this preparation, there are M bands recognizable in both myofibrils, and a conspicuous series of elevations in the shorter myofibril These are contraction bands The residual M bands are still distinguishable between the prominent contraction bands

Porter The whole thing has shifted, though, Dr Hass The M bands are in between the elevations, actually

Hass Yes, they are

Porter The residual M bands

Hass Yes, they are definitely recognizable

Porter But take it down into the contracted area

Hass Here the M bands are in between the contraction bands in both myofibrils but they are less distinct in the more fully contracted shorter myofibril

Porter It is a reaction that begins, undoubtedly, in the Z band region of the I and it is as though you have progressive condensa-



FIGURE 19 Electron micrograph (x 20,000) of rabbit skeleton myofibrils isolated with a colloid mill, suspended in glycerinated buffer solution, contracted at 0° to 8° C with ATP, fixed with formalin and shadowed with chromium In the more fully contracted myofibril the sarcomere length is 50 per cent of that of the average uncontracted mill-isolated myofibril The sarcomere length of the other is at the lower limit of the aver [illegible]
Reprinted, by permission, from [illegible]
Hass, G M Observations by [illegible]
fibrils induced with adenosinetr [illegible]

tion of material from that point toward the M, with the resulting contraction.

Hass: Foreshortening also occurred, but intact I disks and Z bands are not required for contraction. Under these conditions an increased density develops in the A-I region

Porter. Should you not point out that this is an isotonic contraction? If you hold a muscle at its relaxed length and then stimulate, the A band does not disappear.

Hass: That is true. This is a shortening of the whole myofibril



FIGURE 20 Electron micrograph (x 12,900) of a trypsin-isolated rabbit skeletal myofibril which had been suspended in glycerinated buffer solution, contracted with ATP at 0° to 8° C., fixed with formalin and shadowed with chromium The sarcomere length is about 50 per cent of that of the control uncontracted myofibrils Reprinted, by permission, from Ashley, C A., Porter, K R., Philpott, D E., and Hass, G M Observations by electron microscopy on contraction of skeletal myofibrils induced with adenosinetriphosphate *J Exper Med* 94, 9 (1951)

in its long axis, so that actually it is contracted It is not held in extension during stimulation

Figure 20 shows a myofibril in a still more advanced stage of contraction I believe that this was calculated as somewhat greater than 50 per cent contracted All that can be distinguished are the contraction bands, and running through them are the elementary filaments of the myofibril structure These little filaments were of such dimensions in many preparations that they could be measured and Dr Ashley measured over a thousand of them, obtaining their lateral dimensions in the various stages of contraction



FIGURE 19 Electron micrograph (x 20,000) of rabbit skeleton myofibrils isolated with a colloid mill, suspended in glycerinated buffer solution, contracted at 0° to 8° C with ATP, fixed with formalin and shadowed with chromium In the more fully contracted myofibril the sarcomere length is 50 per cent of that of the average uncontracted mill-isolated myofibril The sarcomere length of the other is at the lower limit of the average uncontracted myofibril isolated in this manner Reprinted, by permission, from Ashley, C A, Porter, K R, Philpott, D E, and Hass, G M Observations by electron microscopy on contraction of skeletal myofibrils induced with adenosinetriphosphate *J. Exper Med* 94, 9 (1951)

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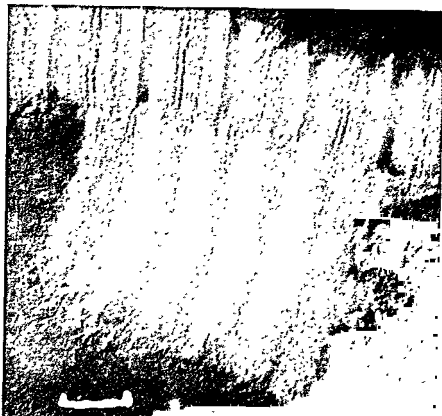


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Figure 20 Electron micrograph (× 12,000) of a [illegible] skeletal

Hass, G M *Observations by electron microscopy on contraction of skeletal myofibrils induced with adenosinetriphosphate* *J Exper Med* 94, 9 (1951)

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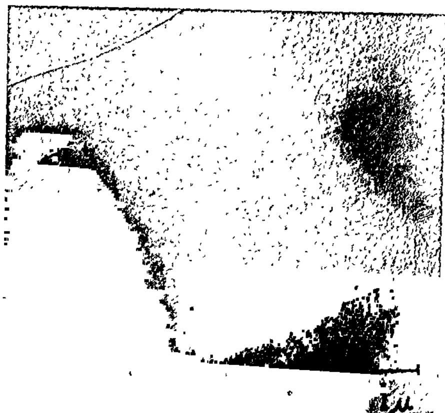
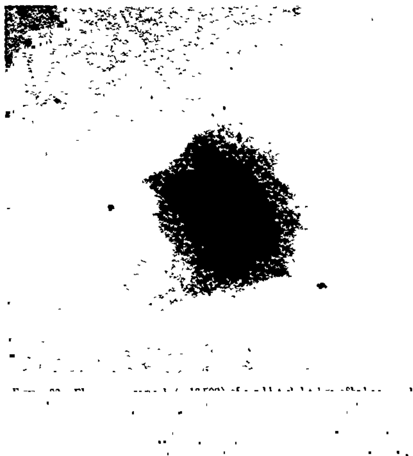


FIGURE 21 Electron micrograph - -olated rabbit skeletal --led -- -n aqueous

keletal myofibrils mauced

Figure 21 shows a myofibril which was 70 per cent contracted, as judged by the average measurements between the contraction bands There is preservation of filamental structure, although it is not quite so regular in this highly contracted stage. The contraction ... cross bands of the myo-

Figure 22 shows the detailed structure of the little spheres that I showed you in the original illustrations. You may recall that the elongated myofibril on the addition of adenosinetriphosphate contracted down to a smaller irregular spherical mass, which was



9 (1951)

depicted as a small dot in Figure 5. This is the structure of the fully contracted myofibril mass as defined by the electron microscope It has lost all semblance of filamentous and contraction band structure, being more or less amorphous, and illustrates the end-stage of an wholly unphysiologic degree of contraction This myofibril was probably 90 per cent contracted.

Figure 23 shows skeletal myofibrils that were isolated by use of trypsin There is fine detail of the structure in the A disks, but the Z bands are missing and there is complete disorganization of the I disks The question arises as to the significance of the attack of

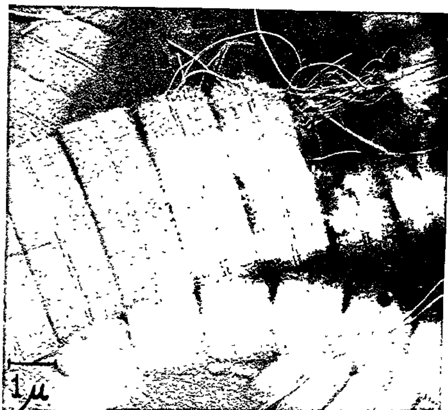


FIGURE 23 Electron micrograph (x 13,500) of trypsin-isolated rabbit skeletal myofibrils, fixed with formalin and shadowed with chromium The I disks are very narrow and the Z bands are absent Reprinted, by permission, from Ashley, C A, Porter, K R, Philpott, D E, and Hass, G M Observations by electron microscopy on contraction of skeletal myofibrils induced with adenosinetriphosphate *J Exper Med 94, 9 (1951)*

trypsin upon the Z band and the I disk Certainly, the enzyme either has some selective effect, or the A disk is relatively resistant to its action The M bands are shown here with the depressions on either side of diminishing electron density, and then a zone of increased electron density, which outlines the position of the H bands.

Figures 24 and 25 show skeletal myofibrils isolated by use of trypsin The I disks and Z bands are practically gone. There is just a narrow space where the I disks should be, and there are no Z bands. Most of that which remains is a series of A disks held together by, I would assume, some residual filamentous attach-

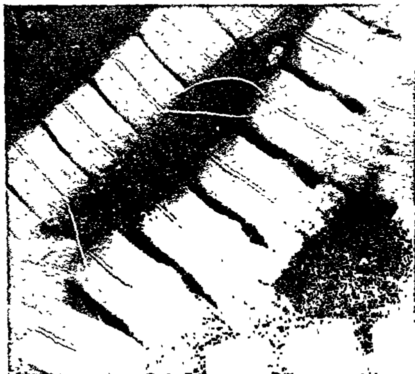


FIGURE 24 Electron micrograph (x 12,000) of trypsin-isolated rabbit skeletal myofibrils, fixed with formalin and shadowed with chromium The narrow I disks and absent Z bands were characteristic findings in trypsin-isolated myofibrils Reprinted, by permission, from Ashley, C A, Porter, K R, Philpott, D E, and Hass, G M Observations by electron microscopy on contraction of skeletal myofibrils induced with adenosinetriphosphate *J Exper Med* **94**, 9 (1951)

ments These were uncontracted myofibrils In the process of contraction, there seems to be a condensation of material which is largely in the A-I disk region Figure 26 shows an early stage of contraction of a trypsin-isolated myofibril

Figure 27 is an illustration which shows the elementary filaments in a trypsin-treated preparation The filaments were measured in length and breadth There were no filaments in trypsin-treated preparations which were longer than the greatest length of an uncontracted normal A disk The filaments varied in diameters from perhaps 50 or 75 up to approximately 200 Angstrom units, with an average of about 175 Å The filaments in the contracted prep

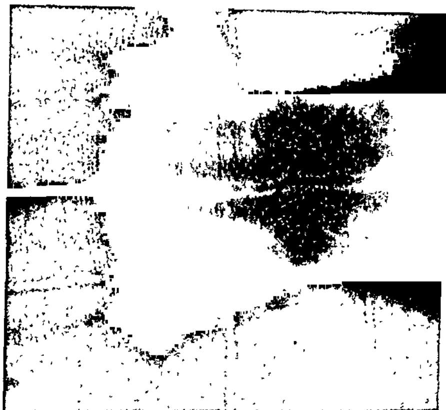


FIGURE 25 Electron micrograph (x 25,000) of trypsin-isolated rabbit skeletal myofibrils, suspended in glycerinated buffer solution, fixed with formalin and shadowed with chromium Only the delicate structure of the A disks with well-defined M bands, H bands and longitudinal fibrous components remain Reprinted, by permission, from Ashley, C A, Porter, K R, Philpott, D E, and Hass, G M: Observations by electron microscopy on contraction of skeletal myofibrils induced with adenosinetriphosphate *J Exper Med* 94, 9 (1951)

tions — and this represents a contracted preparation — varied from about 125 Å up to about 250 or 300 Å in diameter, so that the average diameter of the filaments in the contracted preparation was greater than the filaments of uncontracted preparations (Figure 28). Drs. Porter and Ashley were not convinced that this increment in diameter was due to an intrinsic change in the filament: they believed that the increase in diameter might be due to an excessive adsorption of A substance to the elementary fibrous structure of the filament.

This point has significance from the standpoint of the theory of the orientation of the molecular chains which make up the filament.

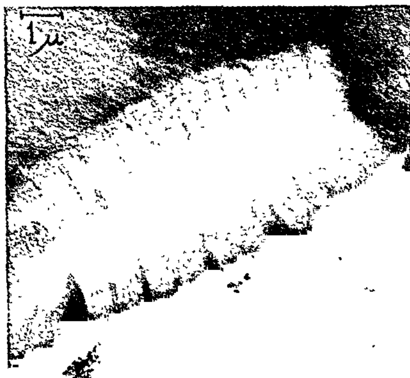


FIGURE 26 Electron micrograph (x 13,500) of a trypsin-isolated rabbit skeletal myofibril, suspended in a glycerinated buffer solution, contracted with ATP at 0° to 8° ... early stag... disks and ... position ... Porter, K ... on contra... *Med* **94**, *9 (1951)*

It is generally believed that the filament is made up of molecules which are polymerized in a long chain, and that the filament rests in the extended state with the chains more or less unfolded In the contracted state, there is assumed to be folding of molecular chains in the elementary filaments This, in itself, might cause increment in lateral dimensions Actually, these measurements, or some of them, were very close to molecular dimensions. I believe that some measurements were about 30 or 40 Angstrom units and, as I said before, the calculated diameters of many protein molecules in the body are about 25 to 30 Angstrom units

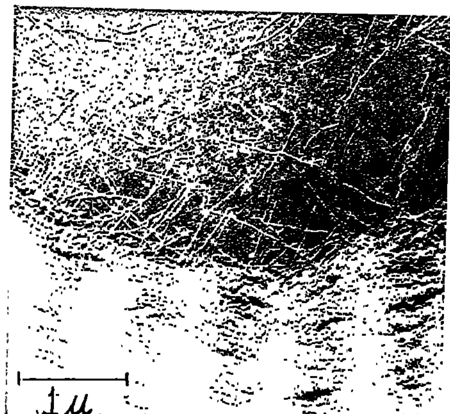
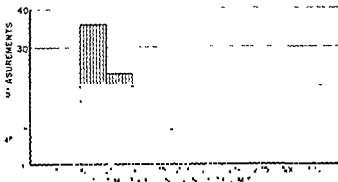


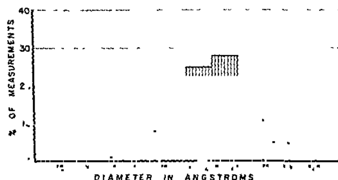
FIGURE 27 Electron micrograph (x 35,000) of the margin of a trypsin-isolated rabbit skeletal myofibril, suspended in a glycerinated buffer solution, contracted with ATP at 0° to 8° C, fixed with formalin and shadowed with chromium The sarcomere length is 55 per cent of the average sarcomere length of control uncontracted myofibrils The interlacing of delicate longitudinal fibrous components obscured only by the increased electron density of contraction bands is shown. Reprinted, by permission, from Ashley, C A, Porter, K R, Philpott, D E, and Hass, G M Observations by electron microscopy on contraction of skeletal myofibrils induced with adenosinetriphosphate *J Exper. Med* 94, 9 (1951)

Figure 28 is a summation of the measurements made by Dr. Ashley, of the diameters of filaments in contracted and uncontracted preparations There is a shift in average measurements which indicates some increment in the basic diameter of filaments during contraction Again, I must emphasize that the filaments have a slightly irregular, nodose form, so that one cannot be certain that only the diameters of compact long molecular chains were measured

Porter. Would it be a good point to inject the observations of Rozsa, Szent-Gyorgyi and Wyckoff (8), that these filaments may



FILAMENTS IN UNCONTRACTED PREPARATIONS



FILAMENTS IN CONTRACTED PREPARATIONS

dissolve out and give particles, or slightly elongated spheres, which will, under certain conditions of KCl concentration, then polymerize again into filaments and show the same periodicity as the filaments in the myofibril? These authors point out that the filaments seem to represent the action component of the fibril

Hass: This will be discussed later on At this time, it might be well to review what is known about the structure of the components illustrated in the electron micrographs. The transverse bands have been demonstrated with the light microscope to a limited degree, especially in preparations that have been properly stained. In the resting intact muscle, the disks and bands of individual myofibrils are coordinated transversely, so that they adjoin one another. There is evidence that the coordination is maintained structurally by continuity of Z bands from one myofibril to the next This evidence derives additional support from the present studies, which indicate that the separation of myofibrils from one another is facilitated by destruction of Z bands by trypsin. The Z bands, in turn, may be connected in some way to the sarcolemma.

There are other details of cytologic integration of structure which were disturbed by the preparative methods The most important of these is the relation of mitochondria to the band structure This relation has been emphasized by Drs Harman and Feigelson, in Dr. Angevine's laboratory (9,10). If Dr. Angevine wishes to, I should like him to discuss the relationship between mitochondrial structure and myofibrils in the intact cell.

Angevine. What I have to say will not take long to present

Holbrook· I have been afraid to ask this question, but I should like to include it in the record before you start, Dr. Angevine, if I may I have been fascinated with the process of reversibility. We haven't any answer to that as yet, but I should like to ask whether you had tried varying the concentration of adenosinetriphosphate If you used one tenth as much adenosinetriphosphate, did you get as complete contracture of the fibril to a little round dot as you did with the amount you selected? In other words, is there a variability with the amount of adenosinetriphosphate and the degree of contraction?

Hass: There is a variability. That is a problem on which we are working, Dr Holbrook, at the present time. If we are to reverse the contraction, as illustrated here, we may have to work with a concentration of reactants which will lead to lesser degrees of contraction. We have evidence which indicates that we can, by control of the amount of ATP added in relationship to the available number of myofibrils, obtain a degree of contraction which is less pronounced than that shown here

Holbrook: That is what I wanted to know.

Hass: This latter approach is one which is contingent upon prior knowledge of the amount of myofibril material in the reaction mix-

ture. The best way to obtain that knowledge is to bring the myofibrils to a dry state and analyze quantitatively. Bringing the myofibrils to dryness destroys the contractile properties. Hence, we are faced with the problem of approximating the minimal concentration of adenosinetriphosphate, which will give a contractile response of an estimated rather than an exact quantity of myofibrils *The problem of mixing of reactants is also involved, for we h*
good evidence that the added aden
tively used up before
time is being conduct
gene

...ally enough?

Fremont-Smith How high do you have to go to get *a contractile* temperature?

Hass: About plus 8° C is suitable Contraction probably begins in the neighborhood of 0° C and becomes evident in the temperature range from 0° to 8° C.

Holbrook It seemed a good question for me to ask, because if there is no contraction without ATP, and the mol concentration *which is used squeezes everything up into a little black ball*, somewhere in that range there might be a degree that could be seen and measured if the technique could be worked out

Hass That is a critical point, and it will mean a great deal, provided it can be shown that there is partial rather than all-or-none contraction of myofibrils I suspect that we may find that all-or-none contraction is not the only possible response of the isolated ... fibrils In any event, observations
under optim...
is requ... ...myofibril If less than this qu... ...available, contraction is incomplete Thereafter, contraction goes to completion when additional adenosinetriphosphate is added to the system The same rules also seem to apply to the quantity of magnesium ions

Meyer· The fibril on top of Figure 19 appeared contracted; one part was not Would one not expect a gradient of noncontracted to contracted fibrils?

Holbrook. The two wide bands we saw – the one below much more contracted than the one above – are the ones I thought were not a trypsin preparation

Porter You are right, that was not

Meyer. In Figure 19 you pointed out the upper fiber. I think the other, the lower one, would best demonstrate it.

Porter One fibril was responsive and the other was not

Meyer: That is a question in relation to adenosinetriphosphate. Why is there no reaction whatsoever? Or do I understand it correctly?

Porter But, you see, the preparation methods might have induced contraction, and so contractility was lost in some fibrils and not in others

Fremont-Smith Some of them have been hurt.

Meyer: That is a difficult conception.

Hass That is true to some extent. We have occasional preparations in which no contraction, or only partial contraction, occurs. However, the conditions under which we work at the present time lead to complete contraction of essentially all myofibrils in the microscopic field

Holbrook: With adenosinetriphosphate?

Hass Yes As I indicated before, the methods of approach to the problem of all-or-none contraction are quite clearly outlined in so far as ATP-induced response is concerned

Travell May I bring up a point which has to do with the preparation of the cardiac and skeletal myofibril? As I understand it, the removal of the sarcolemma by trypsin is necessary in case of the heart fiber, and not in the case of skeletal muscle, and I wondered whether you would comment on the difference between these two fibers, which makes trypsin necessary in one process and not in the other

Hass. I am not enough of a histologist to be sure that the cardiac muscle cell has a sarcolemma The skeletal muscle does have a sarcolemmal investiture, but I don't believe that the cardiac muscle cell does It may have, but it seems to have a perimysium of delicate nature.

Porter That doesn't deprive it of a sarcolemma.

Hass. No, it does not, that is true. Dr. Porter says it has one, so it must have.

In any event, let us review the difficulties with cardiac muscle. In the first place, skeletal muscle is composed of long parallel cells which contain multiple nuclei. They are physically arranged in such a manner that it is much easier to disintegrate structure because of the linear parallel apposition of the muscle cells

Holbrook That makes sense

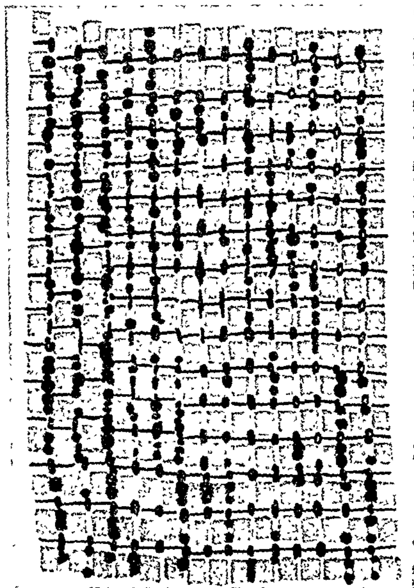


FIGURE 29 Diagrammatic sketch of muscle to show Altmann's granules Reprinted, by permission from Altmann R *Die Elementarorganismen und ihre Beziehungen zu den Zellen* Berlin de Gruyter, 1891

Hass: In the heart, there is a complex arrangement of cells which is in the form of a continuous syncytial rather than parallel arrangement. Furthermore, in cardiac muscle there is, per unit area of cytoplasm, a smaller number of myofibrils than in skeletal muscle. Also, in the cardiac muscle cell there is a larger amount of interfibrillar cytoplasmic material, which may in a cohesive sense hold the myofibrils together to a greater degree than in skeletal muscle. I believe that trypsin acts more in disrupting the protein cohesion of cytoplasm to myofibril than the sarcolemmal investiture That is just a guess, however. All I can say is that we can isolate, in purified form, a great many myofibrils from skeletal muscle without the use of trypsin. It has become routine in the laboratory, and is a daily preparative manipulation. Dr. Schick has worked continuously at the problem of isolating myofibrils from the human and animal myocardium The yields from cardiac muscle without using trypsin are extremely low. He continues to use trypsin to get a respectable yield of myofibrils from the heart

Holbrook: That is clear. Now would you like Dr. Angevine to show us those figures?

Hass: Yes, I should like to see them.

Angevine: We are not quite as highly departmentalized as Dr. Fremont-Smith led us to believe last night. About four years ago, the enzyme chemists came to us for help Dr. Harman, a young man in our department, who was trained in biochemistry before he became a pathologist, was uniquely prepared to do some studies with Dr David Green. Since that time he has been working independently on muscle. I am just going to show rather briefly what his approach was in relation to these studies.

Before coming, I asked Dr. Harman whether he had any figures in his possession that might interest this group This is Figure 29, from Altmann's original work (11), in which he described Altmann's granules The black dots correspond to mitochondria between which are the myofibrils. The black transverse lines connecting the mitochondria probably correspond to the Z lines that we have been discussing this morning. This is added more or less for historical interest, to show what one can do with an ordinary light microscope.

Figure 30 is another schematic representation to show the mitochondria, which appear as large dots. I am showing it to you to point out one thing· although Dr Harman has been principally interested in the activity of the mitochondria, he prepares his suspensions of material in the Waring blendor, and then he examines microscopically the material that he is working with prior to, and

Mbr
mf
Z
S
K
n

FIGURE 30 Sketch of muscle to demonstrate the relation of mitochondria to myofibrils Reprinted, by permission from Meyer, A *Morphologische und physiologische Analyse der Zelle der Pflanzen und Tiere* Jena, Fischer, 1926

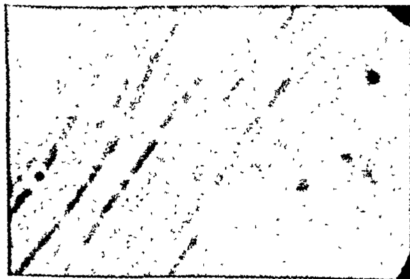


FIGURE 31 Dark rod shaped mitochondria with myofibrils in the background x 1700

after separation of, what he thinks are relatively pure mitochondria or myofibrils. This three-dimensional picture is of some interest, because it illustrates the mitochondria. They are in the space which has been exaggerated, and is probably where most of the soluble enzymes are situated.

Fremont-Smith: Where are the nuclei? On a different level altogether?

Angevine: There are no nuclei seen here. Figure 31 shows one of the preparations of fresh muscle in which the mitochondria, the long, dark, ovoid structures, are prominent. In the background you can see the myofibrils The number of mitochondria depends very much on the type of muscle one is working with, and whether it is red or white muscle. The red muscle has far more mitochondria than the white, and is the more active muscle. It varies considerably in different species, and in any individual species there is a great variation in different muscle. For instance, the leg muscle may have few mitochondria. The smallest number, I believe, are in the gastrocnemius or the soleus. The diaphragm is filled with them, and the heart has still more, so it depends entirely on the preparation which you are working with. It is definitely related to activity: that is why the enzyme chemist works so extensively with pigeon breast muscle Now they are more interested in fly wings and humming bird wings and such things

Heart muscle is the one containing most mitochondria. On the basis of Harman's studies (9,10), he feels that most of the oxidative phosphorylation is situated in these mitochondria, which are very rich in both ADP (adenosinediphosphate) and ATP (adenosinetriphosphate). In studying his preparations, he examines both the mitochondria and the myofibrils and finds very little activity in suspensions of myofibrils I might also say that this work has been done under a phase microscope and that Harman has also observed the contraction and relaxation of muscle fibers under the microscope. These studies will be published relatively soon.

Porter. You said the cell is destroyed when the ATP level is destroyed?

Angevine. I mean the myofibril is destroyed when the ATP level is depleted

Porter. It disintegrates?

Angevine· Yes, it will disintegrate when the ATP disappears. In its normal state it is usually relaxed.

Porter. But what does Harman think — that the mitochondria inject ATP into the fibril?

Angevine. They transfer this material. Most of it is high-energy phosphate, which is combined with the mitochondria, and he believes that it acts in the tissue in between and that it is the trigger that starts the myofibril working, in other words, he feels that after you have extracted the myofibrils from the muscle, it is more or less incapable of much activity He has also described the changes in the shape of the mitochondria in relation to oxidative activity, and they correlate reasonably well, I believe.

Bennett: What is the magnification of that, approximately?

Angevine. It is about 1700 times

Bennett. Taken through a phase-contrast microscope?

Angevine. Yes, it is a fresh fiber

Travell What is the change in shape?

Angevine: You mean of the mitochondria?

Fremont-Smith Yes, with activity.

Angevine: This is probably the normal shape When Harman extracts them, he studies the effect of various ions, and so forth, on them and follows the alteration in shape He has done a good deal of detailed study on the various types I don't believe I can answer that too well It has all been described in the literature

Holbrook. Thank you very much Will you proceed, Dr Hass?

Hass I should like Dr Angevine to say a few words about the relationship between the transversely distributed mitochondria and the A disk In one of Dr Harman's recent papers, he states that in cardiac muscle particularly there is an intimate overlaying of mitochondria transversely in relationship to the A disks of the myofibril units (9) This represents an interesting spatial relationship between the mitochondrial factory and the contractile unit

Holbrook Yes, the assembly unit.

Angevine: I can't make any comment on that except, as you have indicated, he has described it and feels there is some relationship to it

Porter. In our laboratory Palade (12) has noticed the same thing in cardiac muscle that is, that the mitochondria are numerous and are closely associated with the Z bands. He also points out that the internal ridges, or cristae, of the mitochondria are more densely packed in mitochondria within very active cells, such as those of cardiac muscle I should like to point out in Figure 32 that besides the mitochondria there is another component here in the cytoplasm, which represents the basophilic substance We have come to speak of it as the endoplasmic reticulum, or in muscle the sarcoplasmic reticulum It is also closely associated here with the Z bands and



FIGURE 32 Chick skeletal muscle in longitudinal section to show mitochondria (m) (or sarcosomes) and elements of the sarcoplasmic reticulum (sr) The latter constitute a lace work of strands and vesicles which is wrapped loosely around the fibrils except at the Z bands where it is frequently concentrated (× 16,000)

is prominent in the connection between the Z bands and the sarcolemma Actually, in good preparations, the strands of this reticulum seem to be vesicular or canalicular structures, and sometimes they can be seen to form rings around the fibrils at this level. This component of the sarcoplasm was observed by the older histologists in their gold impregnations, and very beautifully pictured Some functional relation with the Z band at this level is clearly suggested (13).

Holbrook: It is interesting, inasmuch as the Z bands are apparently the first to become indistinguishable when contraction takes place

Porter. I think everybody agrees that the contraction may have its inception at the Z band.

Dempsey: I am not clear as to what you said Is the black line transecting the light area in those fibers the Z line?

Porter: Yes.

Dempsey Or are you saying that this is another material that accompanies the Z?

Porter: No, the cytoplasmic element associated with the Z is here at the sarcoplasmic ends of the Z line

Dempsey: Does that stuff penetrate into the myofibril along with the Z?

Porter· No, not definitely, it seems rather to be wrapped around the myofibril.

Dempsey· Then everything there is Z?

Porter. Yes

Dempsey. And the Z line goes across the sarcoplasmic space and connects with the Z disk?

Porter No Only the sarcoplasmic reticulum runs across the sarcoplasm at that level

Holbrook Dr Hass, I think you might as well continue

Hass As Dr Angevine has shown, the work in his laboratory is of considerable importance because of the interest of all cytologists in the significance of packets of activity and their spatial relationships to one another in the cytoplasm of cells In this particular instance, it would seem that the oxidative activity of the *mitochondria resulting in the synthesis of high-energy* phosphate compounds may be a mechanism concerned with maintenance and function of the contractile unit (14) The metabolic *relations* between myofibrils and mitochondria would seem to be matters of importance in the eventual integration of studies of cytoplasmic functions in general, with the contractile function in particular

The polarizing light microscope has disclosed that the A and I disks are both birefringent: the A disks more so than the I disks (15) During isometric contraction this property is not appreciably changed, but if the muscle is allowed to shorten the distinctions between the A and I disks disappear. It is assumed that the anisotropy of both disks is due to the arrangement of the oriented molecules of the parallel filaments, as well as the arrangement of the filaments themselves This alone would not allow for a distinction between the A and I disks unless it is assumed that something in the A disks contributes excessively to the anisotropic property. It is believed that this is probably myosin, which, according to present ideas is composed of long rodlike molecules or micelles, oriented along the filaments in the A disk The filaments themselves are currently regarded as composed primarily of oriented molecules of actin, another major protein of muscle The longitudinal periodicity often demonstrated along the filaments is assumed to represent the actin molecule, or elementary packet of molecules representing the actin micelle. Upon the extractive separation of these components, the myosin, which is presumed to be interfilamentous in position, is removed first. This leaves the filamentous residue which, when dissolved, presumably breaks up in rough accordance to the axial periodicity of 400 Angstrom units into G-actin or globular actin. The excess electron density in the A disk is also probably partly due to other constituents of muscle. Presumably, glycogen is localized here in intact muscle, but it is doubtful that any remains for electron micrography, due to the method of preparing the myofibrils.

Inorganic constituents are also concentrated in the A disk Among these, magnesium and potassium are quantitatively most important, with calcium and sodium in lesser amounts (16) There is some theoretical evidence, based largely on the studies of the affinity of myosin and actin for inorganic cations, that magnesium and potassium are principally bound to myosin, and that calcium is attached primarily to actin, or, as it were, to the elementary filamentous structure (17) These ions are also present in the I disks Here, there is supposed to be no over-all concentration of any inorganic ion, but all exist in relation to interstitial fluids in accordance with the rules which govern the Donnan equilibrium There are, however, probably some local concentrations of ions (18).

Recently, an ingenious use of electron beam microincineration within the electron microscope has indicated that the more volatile elements, potassium and sodium, are located in the Z and M bands,

and in the A substance (18) The less volatile elements, magnesium and calcium, are distributed along the filaments with a long axial periodicity of about 400 Angstrom units There are doubtless many other components, especially so-called associated proteins, in the A and I disks, but little is known about them. Certainly in our preparations, any loosely bound or loosely associated proteins would have been dissolved by the methods of preparation, especially when tryptic treatment was used

The location of high-energy phosphate compounds, which are so intimately connected with the problem of muscular contraction, seems questionable Caspersson and Thorell have localized adenosinetriphosphate by cytophotometric methods in the I band (19) About 0 1 per cent of fresh muscle is composed of adenosinetriphosphate, and its exact location is a matter of considerable interest The reason for this is that ATP in resting muscle is protected, according to some observers, from enzymatic attack. In this regard, Dr. Perry has concluded that there are protein-bound nucleotides, mostly ADP in muscle, and in this state they resist enzymatic attack (20) The enzymatic attack on ATP is initiated theoretically by the excitation impulse, which is assumed to act by depolarization of ionic "membranes" It would be convenient to have the energy source in one compartment, let us say the I disk, and the enzymatic contractible mechanism in a neighboring compartment, the A disk, with a depolarizable "membrane" between them This seems much too simple and hardly substantiated by critical data.

With respect to the composition of the narrow bands within the A and I disks, little is known Future studies of the relation between composition, structure, and function of the contractile unit, doubtless will be concerned with them, for they represent concentrations of materials which appear to change reversibly during contraction and relaxation Some of these changes may be concerned with massive ionic and molecular movements over considerable distances, but this contention remains to be proved

The electron-microscopic study of myofibrils fixed with formalin in successive stages of contraction, induced with adenosinetriphosphate, disclosed changes in structural detail which we have discussed This diagram gives a condensed simplified review of the observed changes (Figure 33) The micrographs raised several questions It seemed clear that the pattern of structural changes, as described during contraction, resembled the probable structural changes which occur physiologically in the contraction of intact muscle (21) The degree of contraction of the isolated myofibrils,

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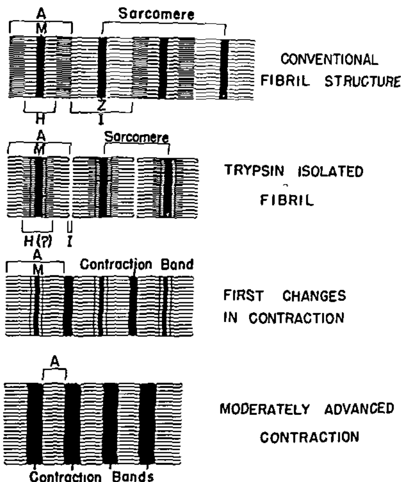


FIGURE 33 Diagrammatic illustration of the changes in the structure of myofibrils during isolation with trypsin, and changes occurring in these myofibrils during ATP-induced contraction Reprinted, by permission, from Ashley, C A, Porter, K R, Philpott, D E, and Hass, G M: Observations by electron microscopy on contraction of skeletal myofibrils induced with adenosinetriphosphate *J Exper. Med* 94, 9 (1951)

however, certainly exceeded anything which occurs in intact muscle under normal circumstances. In fact, the final stages of contraction of isolated myofibrils represented an unphysiological state of complete disorganization from which the probability of a relaxation-return to normal structure would be very slight. In most respects, these changes probably exceeded those which occur in permanent contracture and the delta state of stimulated intact muscle (22).

If further speculation is permitted, especially with respect to the arrangement of elementary fibrils within the individual fibrous components, it may be suggested that the basic molecular arrangement is more like that of a web, as suggested by the criss-cross arrangement of filaments in the disoriented, fully contracted preparations (Figure 27). This possible arrangement of elastic proteins, which retract after extension, has been emphasized by Dr. John Ayer in his studies of elastic tissue.* The extent to which the change in structural arrangement is related to the interactions in solution between actin, myosin, ATP, and salts remains obscure, though there is evidence that salts, rich in magnesium, are concentrated in the contraction band (16)

The electron microscopic observations directed our attention to the chemical aspects of the isolated myofibril-ATP contractile system. It seemed that some interaction of physical or chemical nature occurred between the suspension of myofibrils and adenosinetriphosphate in the substrate. It soon became clear that thoroughly washed and resuspended myofibrils could finally be studied in an essentially protein-free substrate, which by itself had no demonstrable action on ATP However, thoroughly washed myofibrils did not contract in the presence of ATP unless magnesium ions were added to the substrate I wish to emphasize that point. Therefore, the essential components of the system known to be necessary for contraction became myofibrils, magnesium ions, and ATP This structural system was therefore analogous to the soluble actin-myosin-magnesium-ATP system which has been extensively studied by Szent-Gyorgyi and other chemists (17,23 24,25)

From data available from numerous chemical studies of muscle and actomyosin by others, it was assumed that the contraction in the isolated myofibril system was related in some way to enzymes which remained in the structure of the washed myofibrils This assumption was strengthened by the observation that heating isolated myofibrils at 60° C for a few minutes, or the addition of

*Ayer, J P Personal communication

small amounts of copper ions, which inactivate the ATPase and also oxidize protein sulfhydryl groups, resulted in a loss of the contractile response. The reversibility of the action of the copper ions was not studied, although it has been shown by others that oxidized sulfhydryl groups can be reduced and ATPase activity restored by addition of substances such as sodium cyanide or cysteine to the soluble myosin-ATPase systems previously inactivated with copper ions (26).

Dr Ashley and Mr. Arasimavicius then undertook an analysis of the degradation of ATP by the myofibril, and its possible combination with the myofibril, using enzymatic and ultraviolet spectrophotometric methods developed by Kalckar (27). In using these methods, which are designed for microdetermination of the composition of a mixture of ATP, ADP and AMP (adenosinemonophos-

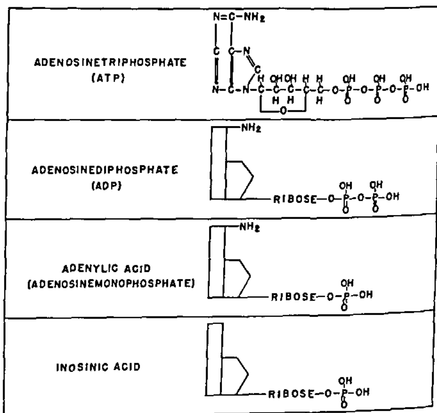
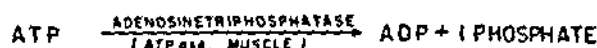


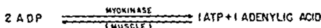
FIGURE 34 The conventional chemical formulas of the compounds analyzed by use of Kalckar's enzymatic-spectrophotometric method.

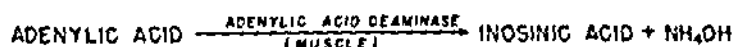
phate or adenylic acid), pyrophosphatase was obtained from potatoes, myokinase from muscle, and adenylic acid deaminase from muscle. Two principles were involved in this method. One was the use of specific enzymes; the other was the measurement of the decrease in ultraviolet absorption at 2650 Angstrom units, as adenylic acid was converted enzymatically into inosinic acid.

This method, worked out by Dr. Kalckar, combines enzymatic and spectrophotometric methods to make an analysis of the composition of a mixture of the compounds shown in Figure 34. The compound "adenosinetriphosphate" contains, at least in theory, three phosphoric acid groups linked together in a chain. These are connected to ribose, and that, in turn, is connected to adenine. The second formula is that of adenosinediphosphate This has the same basic adenine and ribose structure, with only two phosphoric acid groups in the chain The third compound, adenylic acid, as shown in the figure, has only one phosphate group The formula in the lower part of the illustration is that of inosinic acid By deaminase activity, adenylic acid is converted into inosinic acid Now, are the relations between the compounds clear?

Figure 35 illustrates the action of the various enzymes which are implicated in the analysis Through the action of adenosinetriphosphatase, which in this instance is the ATPase of muscle, ATP is converted into ADP plus one phosphate group ADP, in turn, through the action of myokinase, is converted into one molecule







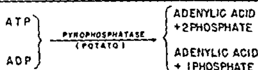


FIGURE 35 The enzymatic reactions involved in analysis of the supernatant fl... of contractile myofibrils by Kalckar's method

of ATP and one molecule of adenylic acid. That is a dismutation reaction. The third reaction involved is the conversion of adenylic acid, by the action of adenylic acid deaminase, into inosinic acid, ammonia, or ammonium hydroxide. The pyrophosphatase obtained from potatoes acts on ADP and ATP, reducing these compounds to adenylic acid, which, in turn, can be assayed quantitatively by the use of adenylic acid deaminase.

The utility of the spectrophotometric analysis depends primarily upon the conversion of adenylic acid to inosinic acid The conversion of adenylic acid to inosinic acid is measured by a decrease in the absorption at 265 millimicrons. These effects are shown diagrammatically in Figure 36. The upper curve is the absorption curve of

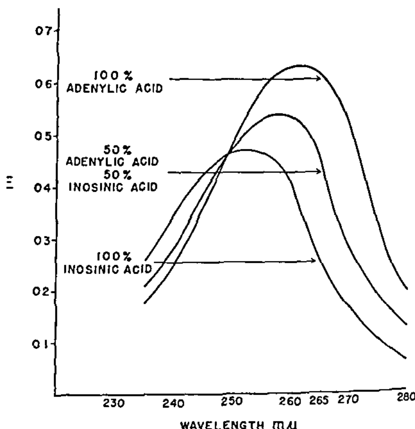


FIGURE 36 The use of ultraviolet spectrophotometric analysis in the determination of the amount of adenylic acid by its conversion to inosinic acid with adenylic acid deaminase

adenylic acid The middle curve illustrates the nature of the curve when adenylic acid is 50 per cent deaminated, leaving a mixture of 50 per cent adenylic acid and 50 per cent inosinic acid. The lower curve is the absorption curve of 100 per cent inosinic acid

In the analysis, the enzymes were added to different tubes (Table I). Adenylic acid deaminase was used for the measurement of adenylic acid content. Adenylic acid deaminase plus myokinase were used to determine amounts of adenylic acid, plus one half of the adenosinediphosphate present, because myokinase dismutes adenosinediphosphate into two components, adenosinetriphosphate and (AMP) adenosinemonophosphate or adenylic acid The third system of enzymatic addition was adenylic acid deaminase, plus myokinase and potato pyrophosphatase This gave a value representing a sum of all nucleotides in the mixture

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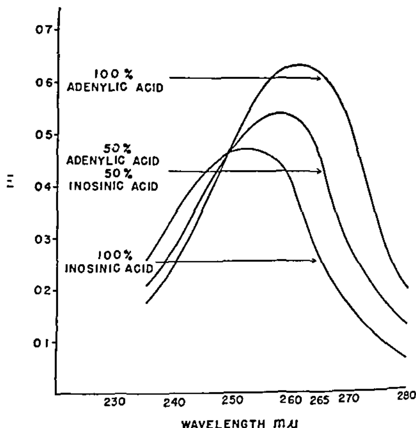


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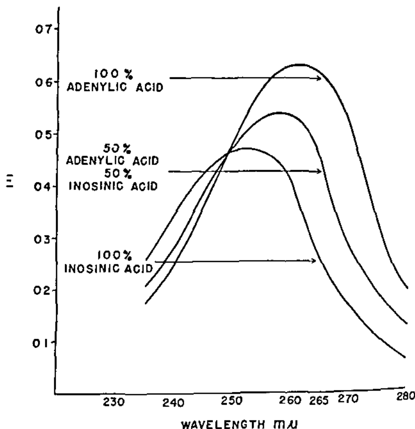


FIGURE 36 The use of ultraviolet spectrophotometric analysis in the determination of the amount of adenylic acid by its conversion to inosinic acid with adenylic acid deaminase.

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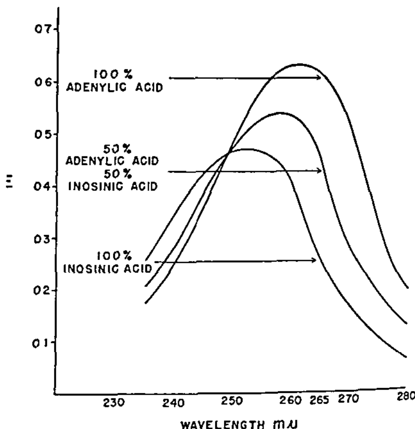


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TABLE II

Averages of Several Analyses Made by Use of Kalckar's Enzymatic-Spectrophotometric Method for the Purpose of Determining the Effect of Myofibrils on Nucleotides Added to Supernatant Fluids

	ATP	ADP	AA	Total
1 ATP Stock Solution	75%	7%	2%	84%
2. Supernatant of Uncontracted with Added ATP	73%	3%	6%	82%
3. Supernatant of Fibrils Contracted with ATP	0%	86%	4%	90%
4. Supernatant of Contracted with Added ATP	42%	34%	5%	81%
Average 2 + 3	37%	45%	5%	–

same in the commercial preparations because of variable degrees of purity. But, by and large, the composition did not depart greatly from the figures shown in the table. Therefore, the ATP stock solution averaged, on the whole, as determined by enzymatic analysis, 75 per cent ATP, 7 per cent ADP, and 2 per cent adenylic acid, leaving a total composition accounted for as 84 per cent nucleotide. When myofibrils were suspended in the precontractile state, supernatant fluid was taken from the myofibril suspension and ATP was added to the supernatant fluid. This was done to determine the effects of the supernatant fluid on adenosinetriphosphate. The data show that the supernatant fluid had practically no effect on any of the nucleotides.

The third line of the table illustrates the composition of the supernatant of myofibrils which had contracted upon addition of ATP The myofibrils used in these experiments had been extracted previously at pH 8, which is a condition we have found effective in removal of myokinase from the myofibril preparations. Myokinase is rather intimately bound to myofibrils, but it can be separated from the myofibrils or inactivated under the conditions stipulated Furthermore, under the same conditions, practically all adenylic acid deaminase was inactivated or removed from the myofibril preparations. Adenylic acid deaminase and myokinase seemed to be separable from the myofibrils, while ATPase remained strongly bound to myofibril structure. The data show that myofibrils prepared in this way converted ATP to ADP, and this occurred, so

far as we were aware at that time, in connection with contraction of the myofibrils.

The fourth line indicates that the supernatant fluid removed from fully contracted myofibrils had no action on ATP, when it was added ATPase remained bound to the myofibril in the contracted state The average of 2 and 3 in the last line of the table indicates values expected if the supernatant of the contracted myofibrils contained no ATPase. Comparison of these averages with the data in line 4 shows that no ATPase passed from the myofibrils into the suspending medium during or after contraction The myofibrils have lost action attributable to adenylic acid deaminase and myokinase It is of interest that there was almost 100 per cent recovery of the ATP as ADP, and under these conditions the myofibrils were contractile That narrows events accompanying the contractile response, therefore, to the conversion of ATP to ADP, in so far as analyses of supernatant fluids of the reaction system are concerned This was a control experiment to determine whether the myofibrils *release an enzymatic component to the substrate after contraction* has occurred The experiment has been repeated again and again We have no evidence that the fully contracted myofibril loses to the supernatant fluid any protein or enzyme during contraction

Porter Does the fully contracted myofibril retain the capacity to split ATP, after having done it once?

Hass We shall arrive at that in just a moment, Dr. Porter. It is a very pertinent question In these experiments, no investigation was made of the possible participation of the enzyme, 5-nucleotidase, which is generally distributed in many tissues and which removes the phosphate group from muscle adenylic acid at an alkaline pH (28) This enzyme has an optimum activity well above pH 8 These determinations were made at a slightly acid pH, in the neighborhood of 6 1 to 6 4 There are other alkaline phosphatases also in muscle We have not studied them, whenever we tried to study the contractile properties of these preparations, or anything else about them at the higher pH values, we had little success

There were three important questions which had to be considered before more concrete ideas about the contractile response could be formulated One question was concerned with the mechanism of the theoretical energy transfer from the adenylpyrophosphate in solution to the myofibril Theory indicated that energy for contraction may be supplied by rupture of the P-O-P (pyrophosphate) bond of ATP and, possibly under some conditions, ADP Presumably, the bond energy could be supplied by direct participation

of the bond in a chemical reaction which involved chemical groups in the structure of the myofibril. Here we have the problem of something apparently not in solution, the myofibril, which has a profound and speedy enzymatic action on a compound in solution It is impossible for me to visualize exactly how this happens.

It brings up the problem of what is in solution and what is not Ordinarily, we think of most reactions as being conditioned by ionic mechanisms, involving materials in solution. Here we are dealing, as in most cellular mechanisms, with a form of chemistry in which the biochemist or the physical chemist has not yet exactly formulated the nature of the reactions. Perhaps Dr. Meyer could help us out at this juncture.

Meyer: No, I really do not see your dilemma We obviously have chemical reactions going on in solid surfaces. We have ion exchange resins where displacement reactions take place.

Hass: A vision of the solid surfaces of the enzyme is the difficulty for me.

Meyer: In biological systems we have solid surfaces which are hydrated. These surfaces allow hydrogenations and dehydrogenations. Take platinum as a model: I assume both hydrogen, and the

and not in solution. Otherwise, it would be difficult to understand step reactions, for example, as in the various cycles The enzymes catalyzing these reactions presumably are arranged in sequence on surfaces. A substrate of the Krebs cycle, for example, presumably travels around over considerable distances

Fremont-Smith: Is electroplating the simplest situation? It is identical, isn't it?

Meyer. Yes, the simplest. I should say that most of our biological reactions take place on surfaces and not in free solution; otherwise, you could not possibly explain any of the step reactions, such as the cycles The substance never loses: that is, the substance to be transformed never gets off the surface. If it does there is some decay: the substance is removed out of the cycle, and there is a certain loss It is not 100 per cent effective, but for all practical purposes all or most of the reactions take place on surfaces, and actually in the molecule, and since these enzymes are all proteins, I think the whole problem of what is solid, what is not solid, and what is in solution, becomes quite academic. That is why they are proteins, in order to allow traveling around a surface, on a surface,

or over large distances The participation and active energy are supplied by the enzyme traveling through this large molecule without any difficulty.

Hass: We must assume, then, that the energy is about 12,000 calories per mol, which is available in the pyrophosphate bond and cannot be transmitted across wide open space. The energy is transmitted through operation of the bond in a chemical reaction, presumably involving myofibril structure

Now the second question was concerned with the timing of the rupture of the high-energy phosphate bond of ATP in relation to the contractile response The temporal relation between contraction and dephosphorylation of ATP seemed important Most evidence indicates that in intact muscle this presumably occurs during excitation.

The third question was concerned with the participation of magnesium ions in the enzymatic degradative and contractile mechanism. What was the importance of catalysis by magnesium? Could magnesium be an essential coupling factor for mediation of energy transfer? It seemed that we were getting beyond our depth in a maze of questions Nevertheless, in seeking answers, it became necessary to resort to quantitative methods and to prepare hundreds of milligrams of myofibrils for analysis by reproducible procedures.

The first series of analyses was concerned with the possibility that the myofibril gained or lost phosphorus or nitrogen during contraction This is, in part, an answer to Dr Porter's question Analysis of the suspending medium indicated that the nitrogen and phosphorus content was the same before and after contraction The only change was an increase in the inorganic phosphorus at the expense of the organic phosphorus, as indicated in Table III. Furthermore, it was found that the myofibrils, before and after contraction, contained the same amount of nitrogen, inorganic phosphorus, and organic phosphorus, per milligram of dry substance.

Table III will give you an idea of the quantities involved In the first two columns phosphorus and nitrogen values are shown in milligrams per milliliter of the supernatant of uncontracted myofibrils This refers to the composition of the initial supernatant fluid surrounding the suspended myofibrils The next three columns show the composition of ATP and the contribution of the amount of ATP used to the phosphorus and nitrogen content of the supernatant fluids

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Hass. A vision of the solid surfaces of the enzyme is the difficulty for me

Meyer: In biological systems we have solid surfaces which are hydrated. These surfaces allow hydrogenations and dehydrogenations Take platinum as a model: I assume both hydrogen, and the substance to be hydrogenated, are absorbed onto the platinum surface. The hydrogenated compound goes back into solution I should assume that most of the intracellular reactions take place on surfaces and not in solution. Otherwise, it would be difficult to understand step reactions, for example, as in the various cycles. The enzymes catalyzing these reactions presumably are arranged in sequence on surfaces A substrate of the Krebs cycle, for example, presumably travels around over considerable distances.

Fremont-Smith: Is electroplating the simplest situation? It is identical, isn't it?

Meyer: Yes, the simplest. I should say that most of our biological reactions take place on surfaces and not in free solution, otherwise, you could not possibly explain any of the step reactions, such as the cycles. The substance never loses: that is, the substance to be transformed never gets off the surface. If it does there is some decay: the substance is removed out of the cycle, and there is a certain loss. It is not 100 per cent effective, but for all practical purposes all or most of the reactions take place on surfaces, and actually in the molecule, and since these enzymes are all proteins, I think the whole problem of what is solid, what is not solid, and what is in solution, becomes quite academic. That is why they are proteins, in order to allow traveling around a surface, on a surface,

or over large distances. The participation and active energy are supplied by the enzyme traveling through this large molecule without any difficulty.

Hass. We must assume, then, that the energy is about 12,000 calories per mol, which is available in the pyrophosphate bond and cannot be transmitted across wide open space. The energy is transmitted through operation of the bond *in a chemical reaction*, presumably involving myofibril structure.

Now the second question was concerned with the timing of the rupture of the high-energy phosphate bond of ATP in relation to the contractile response. The temporal relation between contraction and dephosphorylation of ATP seemed important. Most evidence indicates that in intact muscle this presumably occurs during excitation.

The third question was concerned with the participation of magnesium ions in the enzymatic degradative and contractile mechanism. What was the importance of catalysis by magnesium? Could magnesium be an essential coupling factor for mediation of energy transfer? It seemed that we were getting beyond our depth in a maze of questions. Nevertheless, in seeking answers, it became necessary to resort to quantitative methods and to prepare hundreds of milligrams of myofibrils for analysis by reproducible procedures.

The first series of analyses was concerned with the possibility that the myofibril gained or lost phosphorus or nitrogen during contraction. This is, in part, an answer to Dr. Porter's question. Analysis of the suspending medium indicated that the nitrogen and phosphorus content was the same before and after contraction. The only change was an increase in the inorganic phosphorus at the expense of the organic phosphorus, as indicated in Table III. Furthermore, it was found that the myofibrils, before and after contraction, contained the same amount of nitrogen, inorganic phosphorus, and organic phosphorus, per milligram of dry substance.

Table III will give you an idea of the quantities involved. In the first two columns phosphorus and nitrogen values are shown in milligrams per milliliter of the supernatant of uncontracted myofibrils. This refers to the composition of the initial supernatant fluid surrounding the suspended myofibrils. The next three columns show the composition of ATP and the contribution of the amount of ATP used to the phosphorus and nitrogen content of the supernatant fluids.

In the following three columns the result of adding adenosine-

of the bond in a chemical reaction which involved chemical groups in the structure of the myofibril. Here we have the problem of something apparently not in solution, the myofibril, which has a profound and speedy enzymatic action on a compound in solution. It is impossible for me to visualize exactly how this happens

It brings up the problem of what is in solution and what is not. Ordinarily, we think of most reactions as being conditioned by ionic mechanisms, involving materials in solution. Here we are dealing, as in most cellular mechanisms, with a form of chemistry in which the biochemist or the physical chemist has not yet exactly formulated the nature of the reactions Perhaps Dr. Meyer could help us out at this juncture.

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In the following three columns the result of adding adenosine-

TABLE III

Condensed Table of Different Experiments Concerned with the Relative Changes in Nitrogen and Phosphorus in the Supernatant Fluids of ATP-Myofibril Systems

	SUPERNATANTS														
	Uncontracted Myofibrils		ATP Contribution			ATP + Supernatant Uncontracted Myofibrils			ATP + Heated Myofibrils			ATP + Contractile Myofibrils			
	P	N	P		N	P		N	P		N	P		N Change	
			Inorg	Org		Inorg.	Org		Inorg	Org.		Inorg	Org.		
	0000*	.0013	0037	.0534	.0424	0039	.0621	0423	0037	0615		.0190	.0424	— 0014	
	0000	0010	0031	0508	.0444	.0035	.0581	.0334	0042	0665		0147	.0509	— 0006	
	.0000	0009	0030	0502	.0432	.0037	.0528	0514	.0036	.0589		.0192	.0410	—.0003	
	0000	0011	0039	.0590	.0480	.0030	.0511	—	0099	.0462		.0155	0432	+.0078	
	.0000	0009	0043	0558	0404	0031	0474	—	.0028	.0628	0491	.0154	.0391	+.0072	
	.0000	0019	.0037	0488	.0403	.0040	.0599	—	.0028	.0618	0367				
			0036	0655	.0553	0043	.0566	—							
Ave.	.0000	0011	.0036	0548	0449	.0036	.0556	.0423	0045	0596	0429	0168	.0433	000	

*Values are in milligrams per milliliter

triphosphate to the supernatant of uncontracted myofibrils may be seen This analysis was for the purpose of demonstrating action, if any, of the supernatant of uncontracted myofibrils when it was mixed with adenosinetriphosphate in the absence of myofibrils. You will note that there was no change In other words, adenosinetriphosphate was quite stable in the presence of the supernatant removed from myofibrils. The next three columns illustrate the composition of the supernatant fluid which was obtained from a preparation of heated myofibrils to which adenosinetriphosphate had been added This was a control system in which the myofibrils had been rendered noncontractile as the result of heating at 60° C for 15 or 20 minutes. The values indicate that the heated myofibril contributes nothing to the supernatant, and that the added adenosinetriphosphate, in turn, was unmodified by its contact with the heated system.

The final three columns illustrate the effect of the addition of ATP to the suspension of contractile myofibrils After the myofibrils were removed by centrifugation the supernatant was analyzed The supernatant fluid showed an increment in the inorganic phosphorus, a decrease in the organic phosphorus, and no net change in nitrogen

Porter: What were these fibrils suspended in?

Hass: In a succinate buffer, potassium chloride mixture (1 to 9) with an ionic strength of 0.154 and a pH of 6 1

Porter: Does that do anything morphologically to the fibrils?

Hass. No, they contract promptly in this medium

Porter: Myosin doesn't come out?

Hass. Not at all, as indicated here, unless some has been previously removed in the preparation and washing of the myofibrils Myosin is basically an alkali-soluble protein, which is best removed by solvents with high ion strengths The original procedure of Edsall (23) for recovery of myosin, involved the use of sodium bicarbonate in a system with high ionic strength so that myosin is for all practical purposes presumably insoluble in the medium used However, other data have indicated that myosin may be removed from our myofibril preparation without having to use strongly alkaline media or solutions with extremely high ionic strengths

Angevine Dr Hass, I should like to ask one question that may be rather stupid, but in the columns showing a comparison of the ATP and the uncontracted myofibrils with the ATP plus the heated myofibrils, it doesn't seem to me that the heating has altered it

very much. Would that suggest to you that perhaps they were in a bad way before they were heated?

Hass. Well, this is an analysis . . .

Angevine: I should think that heating would produce some change in normal fibrils. You see the point I make? These three columns do not seem to be too different, and I wonder whether the fact that heating didn't produce any change might suggest that there had been some degeneration or degradation before you started to work with them.

Hass: The point is illustrated in the last three columns where it is demonstrated that the myofibrils are contractile if unheated. Heating destroys the contractility and ATPase action (Table III).

Angevine: Yes, that is true

Hass: Table IV shows data obtained by analysis of the myofibril part of the system. The supernatant fluids were removed and analyzed, as shown in Table III. The myofibril suspensions were removed by centrifugation and then washed four times by centrifugation to remove any contaminating materials which might have been carried along with them. Having to wash materials in this fashion introduced a difficulty, from an analytical point of view, in understanding the effects of ATP, ADP and phosphate on the system. Nevertheless, for purposes of standardized analysis it seemed necessary to resort to this method. This table represents the analysis of the uncontracted myofibrils and the contracted myofibrils. You will notice that they were treated in two ways: We washed them with buffer and then, not being satisfied entirely with that, we followed that washing with an alcohol-ether treatment for purposes of removing as much salt and lipid as possible in bringing the material to a standard state for chemical analysis.

[illegible]

cor [illegible]

am[illegible] myofibrils under ordinary conditions. There was always a residual content of phosphorus. The ratio between the nitrogen and the phosphorus is shown. After washing with alcohol-ether there was an elevation in the nitrogen value, presumably due to removal of lipids and salts from the preparation. The phosphorus value was reduced very little and there was always a small residual amount The contracted preparations show approximately the same values as those obtained for uncontracted preparations. I should like to emphasize that these analyses were made on quantities of myofibrils which were about 30 to 40 mg. (dry). That means that a small

TABLE IV

The Nitrogen and Phosphorus Content of Duplicate Samples of Myofibrils*

No Exp	Uncontracted			Myofibrils			Contracted			Myofibrils		
	Washed with buffer			Washed with alcohol-ether			Washed with buffer			Washed with alcohol-ether		
	N	P	N P	N	P	N P	N	P	N P	N	P	N.P
1	.1314†	0013	103	.1538	0010	154				.1463	0013	114
2	1739	.0013	134	1283	0010	128	1372	0015	91	1336	0011	121
3	.1531	0007	219	.1707	0007	244	1588	0008	198	1642	0006	274
4	1315			.1592	0009	177	.1281	0010	128	1641	0007	234
5	1367	0012	114	1193	0005	239	1930	0010	193	1776	0007	254
6	1556	0011	144	1622	0006	270	1836	0011	167	1633	0007	233
7	1309	0011	119	1567	0008	196	.1284	0012	107	1480	0007	211
8	1368			1478	0008	185	1289	0011	117	1511		
9	1391	0010	138	1598	.0006	266	1435	0010	144	1613	0007	230
10	1423	0010	142	1556	0007	227	1486	0011	135	1568	0006	261
11	1507	0010	151	.1690	0008	211	1519	0010	152	.1773	.0007	253
Ave	1443	0011	140	.1532	0008	209	1502	0011	143	1557	0008	219

* One sample was not treated with ATP The duplicate sample was contracted with ATP Both samples were washed and analyzed in the same way

† Values are given in milligrams per milligram of the myofibril preparations (dry weight)

change in composition might not be detected in this analysis. Had we used tenfold this quantity of myofibrils, the results might have been different, but in dealing with the quantities which we had available, these results were obtained.

Several possibilities were considered in the light of these data: First, the contracting myofibril might have bound phosphorus compounds temporarily on an enzymatic basis, as suggested by Dr. Meyer, and promptly released them, borrowing energy in the bargain. Second, phosphorus compounds might have been bound loosely during contraction and washed away in the preparation of myofibrils for analysis. We thought this rather unlikely because of our recovery figures on analysis of supernatant fluids. Third, phosphorus compounds might have been bound during contraction, and enzymatically degraded during the postcontractile period required for centrifugation and separation of the myofibrils from the supernatant.

Holbrook Did the findings of the increase in inorganic phosphorus, and the drop in the organic, make that point for you?

Hass: We have, as I shall illustrate later, investigated this problem Fourth, phosphorus might have been transferred by an exchange reaction between muscle and the supernatant. We discarded this as a likely possibility because there didn't seem to be enough phosphorus available in the myofibril preparations to make much of an exchange reaction This is, however, still possible, because despite prolonged washing and dialysis, the isolated myofibrils retained about 10 per cent of the total phosphorus found in fresh muscle, as shown by these figures.

Among these possibilities, it seemed likely that if phosphorus in any form were acquired by the myofibril, it was promptly released. On further study of this problem, it was found, much to our surprise, that the fully contracted myofibrils split ATP at about the same rate as the contracting myofibrils (Table V). The continued postcontractile degradation of ATP was not accompanied by detectable further contraction, for contraction had long since been completed. This postcontractile ATPase action seemed meaningless, though it may have significance in the eventual analysis of the recovery relaxation phase of muscular activity. This finding placed us

Porter: Why is it meaningless? Doesn't it mean that the myosin

TABLE V

The Amount of Inorganic Phosphorus Liberated by Precontractile and Fully Contracted Myofibrils From ATP Which Was Added Successively. ATPase Action Was Unimpaired After Contraction.

Exp No	State of Myofibril	Supernatants After Successive Additions of ATP							
		FIRST		SECOND		THIRD		FOURTH	
		P mg/ml		P mg/ml		P mg/ml		P mg/ml	
		Inorg	Org	Inorg	Org	Inorg	Org	Inorg	Org
1	Contractile	0251	0300						
	Contracted			0189	0340	0192	—		
2	Contractile	0143	0530						
	Contracted			0132	0500	0147	.0517	0145	0531

(or ATPase) is still there, acting as an enzyme, and will continue to act as an enzyme until it is denatured, or washed out?

Hass: May I say that our oversimplified structural model of the reactive state of ATPase was conditioned by enthusiasm for the idea that ATPase action might be restricted to a certain phase of configuration of the molecular chains, either the fully extended form or the contracting form, and that the action might cease at the end of the contraction cycle, permitting restoration of ATP to develop thereafter, unimpeded. This is the viewpoint which we had in mind. I built a molecular model which showed the ATPase sticking its reactive center out to attack the ATP when the myofibril was in the extended state, and pulling its reactive center in so as to be blocked spatially as a center of activity in the fully contracted form. In this model the magnesium ion was related nearby to the chemical groups responsible for contraction, rather than ATPase action.

Porter. Why couldn't it normally be just an exhaustion of ATP followed by a resynthesis?

Meyer You see, this is without intermediate relaxation What they tried to do was to transfer the chemical energy of the phosphate bond to the fibril directly in order to cause contraction

Hass: Yes, that's right.

Meyer: As I see it if you have a magnesium group, it hooks onto ATP, the protein on one side, and the ATP on the other Now, the ATP hooks up to an amide group, the phosphate of this hooks up to an amide group, and this makes—what is the name of that instrument: the harmonica?

Porter: Accordion.

Meyer· Yes, it collapses and goes together: this would be the contracted stage When that is reached it would cause the hydrolysis of ATP via the magnesium, which now goes as a chelate compound to where the phosphorus was originally, and forms a very sensible chemical bond. Naturally, this would not explain the excess formation. The machine would be dead, after this happened, unless energy were put in to open it up again, which is what obviously happens *in vivo*, but not in this situation. This would not happen, unless you now made the additional assumption about the ATPase action, which would be totally uncorrelated with the energy exchange. If one assumes that there are different proteins which act, but do not move directly, as we visualize it, but indirectly via a third compound which interacts, then there would be no conceptual

difficulty about the coupling of energy transfer and hydrolysis The hydrolysis is liberation of heat.

Porter: From the pictures of the fibril, it would almost seem that the ATP being added was migrating into the A band, as though it were going along the myofilaments and affecting the contraction that way.

Hass: But it isn't there, when we analyze for it.

Porter. No, possibly because it is destroyed by the myosin

Meyer. It has to come up again, but then the diffusion of such a large molecule, I think, is highly unlikely. It does take place on the surface. I mean, it is then a question of how far in do you call "in"?

Hass: This is a very rapid reaction as well.

Meyer: Oh, yes; it is almost like an ionic reaction

Hass. You turn around and it is all over. There is a high velocity reaction involved here. In turning back to consider the postcontractile ATPase action, Table V illustrates the data These are two experiments. Others have been done, and show the same results, which indicate that the fully contracted myofibrils continue to split ATP. This observation introduces difficulties which arise as the result of a continuous enzymatic decomposition of ATP in this system The experiments show the same successive splitting of ATP by myofibrils in the contractile and contracted state. The myofibrils were thoroughly washed between successive additions of ATP, and contracted fully on the first addition of ATP But the ATPase activity, as shown by the increments of inorganic phosphorus, was approximately the same on successive additions (Table V) We could have continued this indefinitely if the myofibrils had stood up under the repeated centrifugation and washing.

The only clear result at this time was that inorganic phosphorus was not liberated from ATP in significant amounts unless contraction was occurring or had occurred, and that the degree of contraction was apparently related at times to the increment of inorganic phosphorus at the expense of the organic phosphorus in the medium The degree of contraction, in turn, was also related in some way to the availability of magnesium ions, as well as the amounts of ATP added to the system Attention was therefore directed to magnesium

It was found that prolonged washing and dialysis of myofibrils did not remove a residual amount of magnesium and phosphorus, which persisted in quantities of about one-tenth of those present in whole muscle on a dry fat-free basis However, the washed myofibrils did not contract at all in the presence of ordinarily

TABLE VI

The Influence of the Sequence of the Addition of ATP, Calcium and Magnesium to Specially Washed Myofibrils. ATPase Action and the Presence or Absence of Contraction Were Analyzed

Sequence of Addition				Supernatant				Degree of Contraction
Concentration in mg/ml				Experiment D		Experiment E		
5-10	0 4	0 075	0 082	P mg/ml		P mg/ml		
Myofibrils	ATP	Ca	Mg	Inorganic	Organic	Inorganic	Organic	
1				.0002	0026	0002	.0001	none
1		2		0003	0022	0000	.0000	none
1			2	0005	.0036	0000	.0000	none
1		3	2	0008	0032	0000	.0000	none
	1			0069	.0793	0034	.0605	none
1	2			.0209	.0621	0167	.0467	none
1	2	3		.0211	0622	.0172	.0456	none
1	3	2		.0212	.0609	.0173	.0451	none
1	2		3	0346	.0459	0172	.0457	none
1	3		2	.0342	.0454	.0184	.0441	complete
1	4	3	2	.0351	0476	.0186	.0428	complete
1	4	2	3	.0346	0478	0186	0445	complete
1	3	4	2			.0176	.0416	complete
1	2	4	3			0175	.0458	none

adequate amounts of ATP unless magnesium ions were added to the system This consistent observation gave us the opportunity to investigate the role of magnesium in relation to contraction and ATPase action of myofibrils (Table VI.)

First, it was found that contraction occurred in the usual prompt fashion if magnesium ions were added before ATP, or at the same time as ATP If magnesium ions were added shortly after the addition of ATP, no contraction occurred. This was a surprise to us. Furthermore, the supernatant separated from this latter system had no effect on a fresh preparation of myofibrils unless more ATP was added ATP also induced complete contraction when it was added [illegible]

[illegible] ions, or it was rendered inert in some other way

Fremc[illegible]
not belc[illegible]
tions, I [illegible]
glycogenesis *in vivo*, or in liver sections only, when he suspended them in a fluid containing magnesium ions I just wondered whether there wasn't a potential relationship there to the present discussion

Hass I think very likely, because certainly ATP is important, if not essential, in glycolysis and perhaps in glycogenesis High-energy phosphate passes from ATP to glycogen as a step in the process of rendering it susceptible to enzymatic breakdown ATP is an important donor of high-energy phosphate to a great many energy-yielding compounds preliminary to degradation or synthesis, so that the magnesium ion in the system on which he worked may have had a function related in some way to its function in the system with which we are dealing here The relation, if any, is however, not clear to me

Fremont-Smith. The contractility of muscle is, of course, a very special case, and yet it must belong to the general phase of cellular metabolism. Therefore, through this magnesium analogy, we may perhaps relate muscle contraction to general cellular metabolism

Angerine In this connection, magnesium also activates the ATPase in the mitochondria very strongly

Hass I am not implying that adding magnesium ions activates ATPase I am going to illustrate that it does not, at least within the limits of the time intervals involved in these experiments

Angerine I thought you indicated that it did.

Hass I shall illustrate that added magnesium ions influence con-

traction, but not ATPase action as measured over time intervals of a few minutes. We must be careful to distinguish between the enzymatic and contractile properties of myofibrils; they are not necessarily interdependent.

Table VI, with apologies to Dr. Holbrook, is one that has too much data on it, but I shall try to explain it and point out the results of experiments which are being done at the present time. I have selected our last two experiments. The second experiment is not completed from the standpoint of magnesium analyses of the contractile myofibrils, or at least it was not completed when I left. The first series of columns refer to the sequence of addition of elements to the system For instance, 1 means that it was added first; 2 means that it was the second component added; 3 indicates it was the third component; and 4 indicates that it was the fourth component added to the system The approximate amounts of materials in milligrams per milliliter, which were added to the system, are shown at the head of each column: for instance, the concentration of myofibrils in suspension in milligrams per milliliter was 5 to 10 mg; the concentration of ATP was 0 4 mg per milliliter, the concentration of calcium ions was 0 075 mg. per milliliter, and the concentration of magnesium ions was about the same as that of calcium in Experiment D, and about one-fifth as great in Experiment E, although the latter figure is omitted from the table.

The data in the first line show that the supernatant fluids from the myofibrils alone contained very little inorganic and organic phosphorus in either experiment. The second line shows that after addition of the myofibrils to the system, calcium was added. The addition of calcium did not materially change the composition of the supernatant fluid from the standpoint of inorganic and organic phosphorus The third line indicates the sequence of addition with myofibrils, first, and magnesium, second. This combination caused no appreciable change in the phosphorus partition in the supernatant fluid. Under these conditions, as indicated in the last column, there was no contraction of the myofibrils. The fourth line shows the sequence of addition. first, myofibrils; second, magnesium, and third, calcium Analytical values, again, did not change appreciably, and again there was no contraction of myofibrils

The fifth line shows the inorganic and organic phosphorus content of the ATP, added in milligrams per milliliter in the two successive experiments There was little difference in the composition of the two samples of ATP used The sixth line is important. Myofibrils were added first, and then ATP was added to the system

The change in the inorganic and organic phosphorus indicates that ATP was split, that inorganic phosphorus accumulated in the supernatant, and that organic phosphorus was correspondingly reduced in both experiments. Despite this change no contraction occurred In other words, we have a system in which the myofibril will split inorganic phosphorus from ATP, whether it is in the uncontracted state as shown here, or whether it is in the fully contracted state, as shown by data presented a few minutes ago We [illegible] we have no data on the splitting of ATP by the contracting myofibrils

Fremont-Smith: When you say "contraction," do you mean contraction resulting from the addition or contraction previous to the addition?

Hass: Contraction resulted from the addition or the sequential addition In all experiments myofibrils were added first, as the table indicates. The seventh line shows the sequence of addition myofibrils, first, ATP, second, calcium, third The amount of splitting of adenosinetriphosphate was approximately the same as before No contraction occurred. In the eighth line, the myofibril was added first, the calcium second, before the ATP in this line, and after the ATP in the line above it. We permuted various components to find relationships between their so-called activating effects on ATPase and upon contraction. It did not make any difference whether calcium was added before or after ATP, or whether it was added at all. The effects were the same Therefore, we have no evidence that calcium ions in the concentrations used have any effect on ATPase, as judged by the amount of phosphate liberation from ATP during a period of a few minutes Whatever the effect upon the rate of ATPase action might have been, the contractile reaction of the myofibrils did not occur when calcium was added

These next two lines are important. In the ninth line the myofibrils were added first Then ATP was added The tube was then shaken thoroughly to get a complete mixture, and within a few seconds, magnesium ions were added Inorganic phosphorus increased and organic phosphorus correspondingly decreased presumably as the result of degradation of ATP, in both experiments and in approximately equal amounts There was no contraction. We therefore concluded that magnesium ions alone in the system, in the presence of whatever happened to the ATP just before their

addition, were not sufficient to induce contraction of the myofibrils. Had the magnesium been added at the *same time as the ATP*, as we have done often in the past, or added as the magnesium salt of ATP, contraction would have been prompt. The tenth line illustrates that if magnesium were added to the myofibrils before the ATP, the same amount of splitting of ATP occurred, but in this instance there was complete contraction of the myofibrils.

Fremont-Smith: You get complete contraction?

Hass: Yes, there was complete contraction. In the lower remaining lines, results of other permutations of these various components are shown. *We were trying to determine whether there was any* antagonistic effect between calcium ions and magnesium ions. The physiologists have shown that in ordinary muscular activity there is an important role in the balance or the ratios among the various ions in muscle. These ratios have an influence upon excitability and contractility of muscle. From the standpoint of the amount of splitting of the ATP, the ionic ratios did not seem to be important in this isolated system. Calcium did not seem to add or detract from the system in any way. The three lower lines indicate complete contraction, *but in each instance in which complete contraction* occurred, magnesium was added prior to the addition of ATP. The data indicate further that calcium ions did not prevent contraction in the presence of a similar concentration of magnesium ions.

In conclusion, with respect to action of magnesium ions, this is where we stand at the present time: The increase in inorganic phosphorus as the result of splitting of ATP was about the same in the noncontracted and contracted systems. So far as ATP was concerned, the chemical end-result was apparently the same whether *magnesium was added before or after ATP. So far as the myofibrils* were concerned, [illegible] added, together [illegible] was added after [illegible] was added. These observations were suggestive of ionic regulation of contraction with magnesium as an essential link in the mechanochemical coupling reaction between ATP and the myofibril. The actual chemical role of magnesium, however, remained obscure. Though magnesium ions may be concerned only with acceleration [illegible] to theorize that magnesium, [illegible] as ATP, and for myosin as well, may [illegible] appropriately spaced topochemical reactive centers in the contractile protein, and acted as a carrier of the bond energy to the contractile protein molecule,

as ATPase split the high [illegible] l, releasing ADP and [illegible] What a terrible

[illegible] is very beautiful.

Hass: Although we have not been able to demonstrate any antagonistic action between magnesium and calcium, as we had hoped to do, it may be that the ratio between potassium, magnesium and calcium is more important than the absolute molar concentrations in influencing the contractile system. In future experiments, we expect to increase the molar calcium concentrations approximately tenfold and determine again the effect on this system I suspect that if we use the proper molar ratios of potassium, calcium, magnesium and ATP, we shall be able to regulate and control the magnitude of contraction and the rate of degradation of ATP, as well

Angevine Harman feels, as I said before, that magnesium activates the ATPase in the mitochondria, but he has found that calcium apparently activates the ATPase in the myofibril He has not published it, but has said that in his experience he has seen it happen under the phase microscope, and that is exactly what you say happens if you get the proper ratio But he is using the untreated myofibril.

Travell Containing magnesium?

Angevine Yes

Meyer I note, in all your experiments, that calcium can neither replace nor displace magnesium from the reaction

Hass It had no influence in the molar concentrations used over the time interval employed

Meyer I should predict, no matter what concentration you use, that you will not succeed, because thinking of the chlorophyll molecule, the affinity of the magnesium is to a pyrrole ring or pyrrolidine ring, by means of your protein This effect is absent with calcium, and I do not know enough about the atomic relationship between calcium and magnesium Maybe some inorganic chemist could tell us why that is so Obviously, we do not know

Hass Well I am glad to hear that, because the literature is full of references to the antagonistic action of calcium and magnesium ions in various soluble myosin systems We have been driven to this conclusion on the basis of the experiments shown It may be that our system behaves differently from the myosin and actomyosin systems which have been worked with by other people I think it would be worth while to study reaction rates with the possibility in mind that activation of ATPase action by ions is not necessarily

connected in a quantitative way, or in any specific way, with the activation of the contractile response. Our current experiments are concerned with this matter, and may provide more intelligent interpretations of the results

It may be worth while to review briefly a few well-established physiological and chemical observations on muscular activity (29, 30) Physiologic studies of intact muscle have shown that there is a latent period between stimulus and contraction. Heat production begins in the middle of the latent period Relaxation is passive, without significant liberation of heat. The heat liberated in contraction is exactly proportional to the distance of shortening, but is independent of the speed of shortening, the latter being inversely proportional to the load The heat which is liberated is independent of the amount of work done. In reviewing these matters, A V. Hill concluded that the contractile element of muscle must be coupled mechanically with reversible chemical processes by which energy is supplied in shortening, or absorbed in lengthening (29). The rate at which energy is supplied in intact muscle is controlled by tension in the muscle itself, which I think is interesting. I presume that this kind of mechanical coupling to a source of chemical energy may be generally important in some biological activities other than muscular motion.

Meyerhof has summarized his views concerning chemical activity in muscle (30) Among other things, he has concluded that the initial heat of contraction is schematically due to the splitting of ATP, and that the second period of uncompensated positive heat occurs during the delayed oxidative recovery period.

Meyer: Which you have nothing to do with here, in your system.

Hass: Our system shows no mechanical recovery. Meyerhof stated that the chemical phase which utilizes the high energy of the phosphate bond must coincide with, or precede, the onset of contraction. Our present studies are partly concerned with this matter, for it is clear that the energy liberated by the enzymatic splitting of ATP in the isolated myofibril system must either be dissipated as heat, or converted to other uses by the myofibril Thus, the rate and quantity of chemical interaction between the high energy bond and the contractile elements of the myofibril assume first importance. There seems to be a common ground here between biokinetics and chemistry, which may ultimately bear upon such remote problems as the movement of chromosomes in mitotic division

Sandow has stated that the problem of whether the mechanochemical coupling of ATP and myosin occurs before, during, or

after shortening is of immediate significance (31). The heat of shortening, the Fenn effect, indicates a contraction coupling, but other studies indicate a relaxation coupling. It seems apparent to Sandow that if the key reactions are coupled chemically, in which the reactive group of the contractile protein is bound in a fixed position in a chain molecule, we may have either activity or recovery coupling with ATP. In any case, there must be some mechanism which brings the contractile protein and ATP together. If activity energization is the real process, the mechanism would temporally operate following excitation, and prior to contraction If the coupling occurs during the recovery period, it is a form of coupling which is only slightly exergonic, there being little heat of relaxation This would still permit the essentially thermoneutral dismutation reactions of the myokinase type, or phosphorylase reactions, involving creatine phosphate and adenine nucleotides.

In regard to the preceding comments of Sandow, our studies certainly do not define the temporal relationships between the splitting of ATP, and excitation or contraction of isolated myofibrils They do indicate that the splitting of ATP is not a sufficient chemical event in contraction, and that the uncontracted, or fully contracted myofibrils, are equally effective in liberating inorganic phosphorus from ATP over the time interval used They also indicate that the presence of adequate amounts of magnesium and ATP are necessary for excitation and contraction of the isolated myofibrils Whether splitting of ATP is a necessary condition remains to be proved, even though every experiment indicates that contraction will not occur unless ATP is split Actually the possible range of conditions for contraction may be very great, involving rates of reaction, molecular arrangements, and interactions far beyond our present comprehension However, for the purpose of guiding a study of the substrate phase of the mechanochemical coupling reaction, the magnesium ion seems to lie as an intermediate between the contractile elements in the myofibril and the energy supply created by enzymatic splitting of ATP by the myofibril

As we pass from brief physiological considerations of excitation, tension, work and heat through briefer considerations of intermediary, aerobic and anaerobic metabolism resulting in generation of high-energy phosphate, to an investigation of the more distal events in muscular activity, the chemical concepts of Straub, Szent-Gyorgyi, Mommaerts and others should be reviewed and examined (17,21,25) These concepts involve the interaction of extractives of muscle. At this time, the important extractives are actin, myosin, ATP,

magnesium, and other ions. When these extractives are properly added together in solution, a product which undergoes superprecipitation is formed. Deductions concerning the mechanism of contraction have been drawn from a study of the components of this product and the kinetics of its formation and dissociation.

Briefly, and with considerable simplification, there is evidence that the protein, myosin, in its present state of purification, has a molecular weight of about 840,000. The molecules are rod-shaped, being about 1500 Å in length, and 30 to 35 Å in breadth (25). This protein possesses ATPase activity, either intrinsically or by adsorption of the enzyme The myosin is first isolated from the myofibril structure by the use of concentrated salt solutions, at a slightly alkaline hydrogen ion concentration. This leaves a residue of material from which actin is prepared. After complete removal of myosin with the associated ATPase activity, the residue is extracted and a protein, which is known as G-actin, is isolated. This is called G-actin because the molecule apparently is in globular form When G-actin is placed in combination with adenosinetriphosphate, it undergoes, in the presence of magnesium ions, a polymerization into a form which is known as F-actin, or fibrous actin. Fibrous actin, in turn, reacts with myosin to produce a complex which is known as the actomyosin reaction complex. G-actin, in combination with myosin, is formative of only an inert system. F-actin must be the form in which actin is used if it is to produce a system which undergoes the superprecipitation phenomenon It is to be recalled that in order to form F-actin in its reactive form, ATP is involved, and that presumably one mol of ATP, per 60,000 grams of actin, is incorporated in the actin during the period of polymerization. At the same time, one mol of ATP is transformed from ATP to ADP. There is one mol of actin with a molecular weight of about 60,000, and one mol of ATP is incorporated in the polymerization reaction by which G-actin is transformed into F-actin, mol for mol, as I understand it.

Meyer It would be 800,000 divided by 18,000?

Hass: No, 840,000 is the molecular weight of myosin, not actin.

Porter There is a little confusion there, because the G-actin is a very sizable molecule. How did they arrive at a molecular weight of 18,000?

Hass. It occurs to me that there is some discrepancy in opinions with respect to the molecular weights of the products involved Szent-Györgyi gives one set of figures with respect to molecular weight of the components used in his treatment of the subject (17). As I recall, Mommaerts, in his last publication, gave the figure of

about 840,000 as the molecular weight of myosin, and he did not give a definite molecular weight of actin, indicating that it may be a mixed species (25).

These isolated systems, which operate in solution and which give interesting precipitating reactions, or which, when drawn out into a thread, give certain contractile reactions on the addition of ATP, are of great interest, but there still seems to be some difference of opinion as to the action of ATP on the actomyosin complex. There is one school of thought that believes that actin and myosin become associated in the presence of ATP. The last interpretation which is given by Mommaerts is that ATP produces a dissociation of actin and myosin (25).

Fremont-Smith. What was Szent-Gyorgyi's viewpoint?

Hass. I believe he favored the association theory Mommaerts now believes that ATP produces a dissociation of actomyosin into actin and myosin.

Meyer: That means it would not be connected with the contraction.

Hass: I do not believe there is any specific statement in his most recent paper that defines exactly the relationship between the ATP, actomyosin, and the contractile reaction

Fremont-Smith Szent-Gyorgyi did have a relationship in his theory, didn't he?

Hass Szent-Gyorgyi has apparently established a relationship in his system. There can be no question that the products which have been obtained, and which they have studied in solution, are very useful from the standpoint of building a concept of the chemical phases of contraction

Fremont-Smith: By "they" you mean whom?

Hass. I refer to Szent-Gyorgyi, his pupils, and many others who have worked with the soluble system of muscle proteins However, experience, by and large, has indicated that most synthetic products which become integrated again artificially after a solution of the natural structure, do not behave in the same way as the original natural integrated systems. That has been the experience with collagenous products that have been reconstituted, especially in Dr. Schmitt's laboratory It has been a more immediate experience in the reconstitution of elastic fibrils, as carried out by Dr John Ayer for a time in our laboratory Whether these products are "natural" or near natural, perhaps, is not important Certainly, they provide a better opportunity to gain insight into the possible

chemical behavior of the natural products. Actually, the isolated myofibril system is not, by far, a natural system.

In conclusion, some unduly premature speculation about the possible nature of mechanochemical coupling of ATP and contractile elements of the myofibril, may be permissible. Some imaginary structural model is usually helpful, if it serves only to assist in the design of future experiments. A model useful to us shows the contractile proteins, with at least two essential templates of reactive groups One is the phosphatase template, and this template fits the ATP molecule, irrespective of the stage of contraction It provides continuous ATPase activity in the precontractile, contractile, postcontractile, and presumably relaxation-recovery phases, though nothing is yet known about the last. The second template fits the magnesium salt of ATP, presumably equally well throughout all stages of muscular activity This template, however, provides in some form a firm adsorption, or bonding, by virtue of coordinated relations between ATP and the contractile protein Presumably, this is the nature of the precontractile union, and magnesium polynucleotide relations may be established there with magnesium, as the coupling ion, between adjacent molecules of ATP, and the special template of the contractile protein. After this union is formed, ATPase acts, and the energy of the terminal bond of ATP [illegible] bond [illegible] suited [illegible] ium is likewise set free is a matter now under investigation.

This is a crude model, which must seem particularly unscientific to chemists and physiologists It may, however, lead to a better one, and I should like at this time to show a schematic illustration which should draw together for us many of the things which we have talked about today Figure 37 created a great deal of amusement in my laboratory, which was justifiable and I hold no resentment. This is the view which may be gained from a large number of studies of the elementary myofilament reaction system. One part of the structure represents one longitudinal fibrous component of the myofibril which was illustrated in the electron micrographs. It shows just one of the little filaments, which is about 150 or 200 Å in diameter

I have drawn only a part of the A disk length, indicating the nodose periodicity along filaments which is approximately 400 Å from peak to peak, as was demonstrated in the electron micrographs. This drawing has been condensed so as to show the M band It has

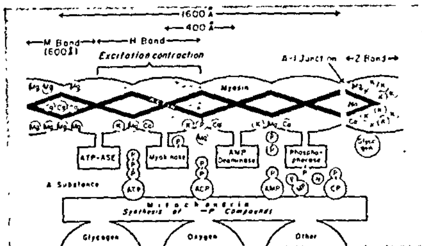


FIGURE 37 Elementary myofilament reaction system This drawing depicts some functional, structural and chemical relations for consideration in reviewing some current ideas about muscle

also been condensed to show the A-I junction, and the Z band has been drawn in close to the A-I junction so that it can be placed on the same figure. The H band is depicted to one side of the M band, and there is a depiction of an excitation-contraction event proceeding toward the A-I junction from the margin of the H band

The material, which lies outside and which occurred as nodosities along the course of the fibrous components, has been designated as myosin. The material which is designated axially and which appears as a nearly linear component of uniform diameter, after myosin has been extracted, has been designated as actin, the internal fibrous molecular chain of the longitudinal fibrous component I cannot say exactly why the internal molecular chains were drawn in this way except that it seemed reasonable that the molecule should be capable of folding in the manner shown The arrangement may be regarded as double helices, or as a meshwork The spacing of the angles along the chains is approximately the calculated length of the actin molecule, about 400 Å Spacing which is indicated above is the approximate length of the myosin molecule, or 1600 Å

The distribution of the electrolytes in this system is roughly shown in Figure 37 The data given by Draper and Hodge, as a result of microincineration studies, indicate that the Z band is largely composed of the more volatile ions – potassium and sodium (18).

The M band also contains potassium and sodium in considerable amounts, but there is, apparently in the myosin, a concentration of magnesium ions According to Szent-Gyorgyi the calcium ions are more intimately connected with the actin framework of the myofibril structure, but I have indicated some general partition of ions along the way without giving them any specific compartmentalization, simply to indicate that there is apparently a high concentration in myosin of magnesium ions.

The part of the structure which is shown below the actomyosin complex is the A substance area between the myofilaments This is the area in which there are apparently many enzymes, associated proteins and other materials. Then, outside the A substance area of *myofibril structure, we have drawn the mitochondria which were* discussed by Dr Angevine. These lie between myofibrils. The mitochondria are engaged here in the synthesis of high-energy *phosphate compounds, and utilize many materials, especially phos*phate, glycogen, and oxygen in the synthesis Anaerobic synthesis may also occur, I suppose, at times. The high-energy phosphate compounds which are generated by activity of the mitochondria, or perhaps other activities with which we are not entirely familiar in muscle, are illustrated here as creatine phosphate, adenosinediphosphate, and adenosinetriphosphate. Adenosinemonophosphate, which in itself has no available high-energy phosphorus, is also shown Creatine phosphate carries a high-energy phosphate. Through the action of phosphorylase enzymes, the high-energy phosphate of creatine phosphate can be transmitted, as shown, to convert adenosinemonophosphate into a diphosphate or triphosphate. Thus, creatine phosphate becomes important in muscular activity, especially in the synthesis of the high-energy phosphate compounds which are illustrated to the left In the figure the high-energy phosphate compounds are built up in one direction, and degraded in the opposite direction

ATPase is firmly bound to myosin as indicated. There is a question as to whether ATPase is actually a part of the myosin molecule. That question has been raised particularly by Meyerhof, who recently stated that there was some evidence that ATPase of muscle had been partly separated from myosin There is another observation which indicates that ATPase might not be intimately connected to myosin. It has been shown that myosin will adsorb potato pyrophosphatase very strongly Hence, there may be no intrinsic molecular composition of myosin related specifically to ATPase function.

Myokinase is another enzyme which lies in close relation to

myofibrils, presumably in the A substance. Myokinase can be removed from myofibrils by continuous dialysis, and by treatment at pH 8. Myokinase is concerned in the dismutation reaction by which 2 mols of ADP are converted to one mol of ATP and one mol of AMP, as indicated Adenylic acid deaminase is a more soluble enzyme of muscle and presumably lies somewhere in the same interfilamentous substance It is concerned with the deamination of AMP, yielding inosine monophosphate, which is an inert compound for our purposes here Inosinetriphosphate is also inert in this system though there is evidence that it is degraded by ATPase.

This figure shows that magnesium joins high energy phosphate to the contractile substance ADP is released at this point, as excitation and contraction are initiated. Just what the nature of this magnesium bond might be is speculative There may be many intermediary activities between the point of action shown, and the actual contractile reaction Data, thus far, indicate that the contracting myofibrils do not permanently bind any products derived from the degradation of ATP The figure illustrates most of the points which have been discussed today. The accuracy of the model is not as great as most models Certainly, it does not depict the true situation, but it may serve a useful purpose to the memory.

In conclusion, I should like to say that the excursions which we have made have not permanently diverted our attention from the original objective, namely, the study of myofibrils isolated from normal and abnormal hearts In recent months, Dr Schuck has improved the methods for isolating cardiac myofibrils The yields are still too small to permit all the types of study which have been discussed However, they are adequate for the application of some analytical methods worked out with skeletal myofibril preparations A matter of greater significance is the proof that human cardiac myofibrils, even though they are not isolated until several hours after the death of the patient, contract completely under the same conditions as myofibrils isolated immediately after death This will, in the future, provide an opportunity for investigating the elementary contractile units of the normal and the failing myocardium As soon as a better insight into the rules which govern the behavior of the skeletal myofibril-magnesium-ATP contractile system is gained a study of the cardiac myofibril may be undertaken

Travell How about rigor mortis of the skeletal muscle, and what stage of contraction does that represent?

Hass It has not been analyzed by us We have attempted to

work with skeletal muscle in the freshest possible condition. The muscle has at times been frozen *in situ*, and at other times immediately after sacrificing the animal. The problem of rigor mortis in cardiac muscle has not been studied. I am sure that you are conversant with the fact that isolated heart preparations have been made and contraction has been reestablished in the hearts of animals which have been dead for several hours. Continued rhythmic activity has been carried on for several days under these conditions. We have no reason for believing that simply because a patient stops breathing, his heart muscle is dead.

If you are interested in the ideas of Lindberg and Ernster, it might be worth while to consider the mitochondria again for just a moment (14). Figure 38 was borrowed from a recent article

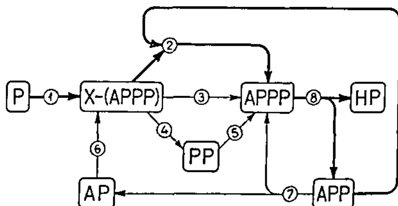


FIGURE 38 This diagram, according to Lindberg and Ernster, illustrates reaction pathways in the cyclophorase-hexokinase system involving mitochondrial activity. Reprinted, by permission, from Lindberg, O., and Ernster, L. On the mechanism of phosphorylative energy transfer in mitochondria. *Exper Cell Research* 3, 209 (1952)

concerned with the activity of the mitochondria This is the system which they have evolved in explaining analytical findings They believe that phosphorus is incorporated by reaction 1 through a process of oxidation which permanently binds oxygen and hydrogen, and is irreversible to form a so-called primary ester. The primary ester, on acid hydrolysis, yields ATP. Apparently very little is known about it – perhaps there are many primary esters – and it may pass a high-energy phosphate to ADP by reaction 2, so that ATP is formed, as shown That is one possibility. The second possibility

by which ATP may be formed is by splitting the primary ester and directly converting this split product into ATP by reaction 3 The third possibility is through an inorganic pyrophosphate compound, which apparently, by reaction 5, reacts with dinucleotide, and ATP is the product. ATP is degraded enzymatically in reaction 8 and, in the presence of a hexokinase system, phosphate is trapped by hexose. Hexose phosphate is formed as a product of the reaction. ADP, in turn, through myokinase reaction 8, is converted into a mol of ATP, and a mol of adenylic acid. Adenylic acid, by a reaction which is apparently unknown, is used in the reformation of the primary ester by reaction 6

The steps in the cyclophorase-hexokinase system are discussed by Lindberg and Ernster and their paper makes very interesting [illegible]

Angecine: I should like to ask Dr Hass one final question When I first asked whether this was contraction or not, there was little comment. What would your reaction be to the following statement? "Nothing has happened here, but you have exhausted the ATP in the myofibril, and it has gone into a state of rigor."

Hass: It is always difficult to put experimental data obtained by study of an isolated system back into the integrated pattern of normal cellular function In any biological experiment, one seems to be confronted with the dilemma of attempting to analyze the hopelessly complex integrated system, or to take it apart slightly and to analyze the activity of its parts I think, however, that in taking it apart, one should do so gently, if possible, because the likelihood of the experimental results having some meaning in terms of integrated cell activity is somewhat greater than if one takes it apart in a rough fashion I am, however, entirely convinced on the basis of what has been learned from all the physiological and chemical work which has been done on muscular activity and metabolism, that the data presented here have a semblance of meaning in relation to physiological reactivity of the intact cell

Holbrook: Dr Hass is carefully avoiding saying that this is a physiological process which occurs

Meyer It would be I don't know whether I understand you aright. In preparing your fibrils and washing them with this buffer and so on, how many of the proteins isolated in solution by Szent-Györgyi and his school, Mommaerts and others, have been [illegible] out? Do you know that?

Hass: I should say, if I understand their methods or procedure of isolation, that very little, if any, myosin or actin has been removed by the preparative methods. I say this because removal of myosin is usually done by extraction with solutions of high ionic strength Ordinarily, ionic strengths of 0 4 and 0 5, or even greater, are used in the separation of myosin from whole muscle. Furthermore, as a rule, as outlined originally by Edsall in his studies, alkaline solutions were routinely used in the preparation of myosin Actin, on the other hand, is a product which apparently cannot be isolated in a satisfactory way, until myosin has been removed by the preparative method which I have outlined Then actin is removed as a water-soluble product from the residue, which has been treated with acetone. I suspect that the associated proteins — a great deal of magnesium and phosphorus, the phosphorylase enzyme systems, adenylic acid deaminase, and in the later preparations, myokinase — whatever their function may be, have been removed by our preparative methods. Mitochondria, of course, are removed very early in the isolation procedure, or are destroyed by the procedure I have hopes that with a little attention to detail, relaxation of contracted myofibrils may be induced I have a feeling that the relaxation phenomenon in muscle is primarily a passive event, and that we may be able to produce a passive relaxation of partly contracted myofibrils if we can obtain a degree of partial contraction which may be reasonably susceptible to reversibility.

Fremont-Smith Do you have to have physical tension on it to get relaxation, and would you be able to demonstrate relaxation?

Hass· The problem of the actual influence of deformed tissue structure on the relaxation phase of muscle has often been debated However, we think that in view of the organization retained in the structure of partly contracted isolated myofibrils, the possibility of obtaining a return to normal configuration is not entirely imaginative It is to be remembered that the pattern of structural change accompanying ATP-induced contraction is very similar to that occurring in muscle which has been normally stimulated to contract.

As was perfectly clear from our experiences with the uncontracted fibril, there is plenty of opportunity for ATPase activity to provide energy from available ATP in the fully contracted state, or in the uncontracted state. The question of coupling energy released by ATPase action on ATP to the partly contracted myofibril is something, again, which has to be taken into consideration In other words, let us take this view Let us say that it might be possible, in the resting muscle, that ATP is not protected against ATPase

action, that ATPase acts continuously, and that we have a constant degradation and resynthesis of ATP. That is the machinery, the power supply, which may not necessarily be contraction-coupled

Porter: But the heat released should become apparent, physiologically, shouldn't it?

Hass. If you deal with dismutation reactions entirely, that is apparently not a matter of very great importance. Likewise, there is a continuous resting metabolism In resting muscle, there is continuous oxidative activity concerned in part with supplying energy for the synthesis of high-energy phosphate compounds In an anaerobic situation, muscular activity is limited to the number of contractions which can be made under those conditions until ATP is exhausted With the aerobic situation, there is normally a continuous oxidative activity, so that determinations of heat liberation on contraction should be done with a control of resting muscle, and an active muscle in the same calorimeter In all normal cells under physiologic conditions, there is a continuous oxidative activity which is concerned with something, but what that is remains a matter of doubt Certainly, replenishment of ATP is one of the concerns

How can ATP be added to a partly contracted myofibril under appropriate conditions, and have it react with a template system which extends the molecular chains rather than contracts them? The energy supply can be put in the system out of a test tube The energy is continuously released from ATP, according to our figures, by a continuous ATPase action irrespective of the state of contraction of the myofibril, so that we can always add the energy supply to the test tube If the energy supply is ATP, the action of ATPase should make some of it available for contraction under one set of conditions, and perhaps for relaxation under another set

Meyer But, you see, you just degrade ATP and nothing happens

Hass: Unless we gear it to the myofibril with magnesium, then there is contraction.

Meyer No, you have magnesium added and you add more ATP Still, the state of contraction does not change You degrade your ATP That means you have added new energy, but nothing happened You don't pull the machine apart

Hass I am not sure, Dr Meyer, that we can't remove the magnesium from the system and I am not entirely sure that something wouldn't take its place in the relaxation reaction There is no harm in trying

Meyer. To remove magnesium?

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Fremont-Smith: Do you have to have physical tension on it to get relaxation, and would you be able to demonstrate relaxation?

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Hass I am not sure, Dr Meyer, that we can't remove the magnesium from the system and I am not entirely sure that something wouldn't take its place in the relaxation reaction There is no harm in trying

Meyer To remove magnesium?

Hass: Yes. It may not be so simple, but nevertheless we shall try to reverse the contraction

Meyer: Have you added versene?

Fremont-Smith: Added what?

Meyer: Ethylenediaminetetraacetic acid. It is called "versene"

Hass Tetraacetate?

Meyer: Yes, which binds calcium and so on.

Hass. No, we have not, but we shall consider using it, if you think it advisable.

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THE EFFECT OF VITAMIN A ON ORGAN CULTURES OF SKELETAL AND OTHER TISSUES

HONOR B. FELL
Strangeways Research Laboratory
Cambridge, England

THE EXPERIMENTS* I should like to discuss this morning were done in collaboration with Sir Edward Mellanby, of the National Research Institute in London For many years, Sir Edward has been interested in the action of vitamin A on the skeleton in animals, and he thought that the organ culture methods that we had developed at the Strangeways Laboratory might be applicable to the elucidation of some of these problems; so at his suggestion, we entered into a collaboration We began by studying the effect of the vitamin on explants of skeletal tissue (1), and having obtained some rather striking results with this material, we have recently transferred our attention to its action on epithelia, particularly the ectoderm (2). I venture to include the account of these experiments on epithelia, even though they are not directly concerned with connective tissue, because I think one should take these results into account when trying to evaluate the effect of vitamin A on the skeletal material.

First, perhaps I had better say a word about the methods, because the kind of tissue culture that we used may not be familiar to people here Figure 39 shows the skeletal explants; some of the epithelial tissues were grown by this method. It is what we call the "watch glass" technique The vessel consists of a Petrie dish, carpeted with wet cotton wool There is a hole in the center of the cotton wool to permit transillumination, and over the hole is laid a watch glass The culture medium is deposited in the watch glass and allowed to clot, and the explant is laid on top of the clot Then, every two or three days, the tissue is removed from the clot and transplanted to a freshly prepared vessel The culture medium

*These investigations were supported by the Medical Research Council and the Nuffield Foundation

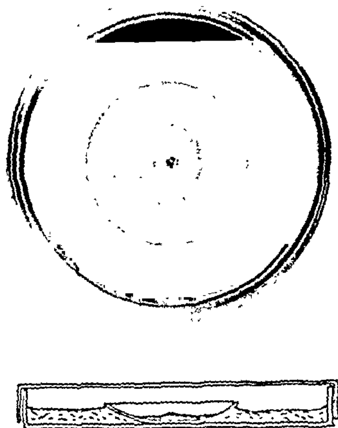


FIGURE 31. Top view and section of watch glass culture

we used *for these experiments* was a mixture of three parts of fowl plasma, to one part of very concentrated extract of thirteen- to fourteen-day chick embryos. Some of the skin explants we grew by ordinary hanging-drop methods, using a large one-inch cover slip and taking care not to disturb the interior of the tissue.

The cotton wool was saturated with sterile distilled water. We put about 10 ml. of water into the dish. The vitamin A was added to the plasma in an alcohol solution of either the acetate or the

alcohol, and the exact amount of the vitamin present in the plasma after this addition was estimated. Of course, the same quantity of ethanol, without the vitamin, was added to the normal control plasma. In all our experiments, the explants from one side of the chick were put into the plus A medium, and the explants from the opposite side of the same embryo were put into the normal medium

I shall deal first with the experiments on skeletal rudiments When a young animal is fed on an excess of vitamin A, very pronounced changes are produced in the skeleton. These have been described by Collazo and Rodriguez (3), and many others, while the histopathology of the changes have been well described by Wolbach (4). The cartilage in the bones is very rapidly consumed, there is extensive absorption of the bone, and spontaneous fractures appear. It was uncertain for a long time whether this effect of the high vitamin on the skeleton, and the opposite effect of the subnormal amounts of vitamin A on periosteal growth (5) were directly on the skeletal tissue or whether they were mediated through, perhaps, an endocrine gland. One of the objects of our investigation was to see whether vitamin A did have an effect on the skeletal tissue explanted in culture, because, under those conditions, the systemic influences are completely eliminated

Holbrook. Dr. Fell, may I ask something about the magnitude of the dose of vitamin A that will produce this sort of thing? Is it roughly huge amounts per unit of body weight? I ask because it has a very pertinent relationship to certain stock practices in this country.

Fremont-Smith: You mean for cattle?

Holbrook: Yes

Fell. Each day the rat in our experiment was fed by mouth, rat cubes plus 40,000 IU (International units) of vitamin A alcohol in avoleum, a commercial liver preparation.

Holbrook: 40,000 units is not a huge amount.

Travell· For how long?

Fell. For 37 days.

Holbrook That is a daily requirement for one cow?

Dempsey: But this is a little rat.

Holbrook: Yes, I know.

Travell· This is half a kilo

Fremont-Smith. Were they young rats?

Fell: Yes, they were young rats: 4½ weeks old at the beginning of feeding

Holbrook. 40,000 units will do that?

Fremont-Smith: Was this a vitamin A mixture?

Fell: Yes, a preparation rich in vitamin A.

Fremont-Smith: It wasn't a single purified A?

Fell. Not for this purpose, no.

Holbrook: This is not synthetic vitamin A, then Was it natural-source vitamin?

Fell: Yes, but it has been produced by the purified vitamin A, by other workers.

Meyer: It is not a mixture of A and D?

Fell. No, I believe not. But that is a standard effect I just discussed it to illustrate what you obtain in animal experiments with vitamin A. In our *in vitro* work, we did two groups of experiments: The first was to study the effect of the vitamin on the differentiation of the skeletal rudiments, and the second the effect of the vitamin on bones that were already at an advanced stage of development when they were explanted I shall deal first with the experiments on the effect of vitamin A on the differentiation of the bones



FIGURE 40 Femur from a 6-day chick embryo. Hypertrophy of the cartilage cells [illegible] bone has formed. x 19 [illegible] ...ion, from Fell, H. B., [illegible] ...ryonic limb-bones cul- [illegible]

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FIGURE 43. Femur from a 6-day chick embryo. Hypertrophy of the cartilage cells is just beginning in the middle segment of the shaft, but no bone has formed. × 18. [illegible] Reproduced by permission, from Fell, H. B., and Mellanby, E.: The effect of hypervitaminosis A on embryonic limb-bones cultivated in vitro. *J. Physiol.* 116, [illegible] 1952.

For this work, we used the long bone rudiments from six-day chick embryos Figure 40 shows the femur of a six-day embryo Hypertrophy is just beginning in the middle of the shaft, but not to any extent There is no ossification at present, and it is very undifferentiated. We isolated it from the surrounding tissue, leaving a little attached, and explanted it by the watch glass method When that is done, the bone grows in normal medium to, perhaps, four times its original length, it differentiates histologically and ossifies, and even acquires, to a considerable degree, its normal shape.

Fremont-Smith Over how long a period?

Fell. I have grown them for three weeks. By then, they usually begin to get rather necrotic in the interior, but it will become quite

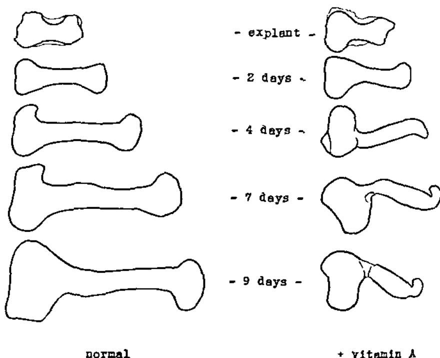


FIGURE 41 Camera lucida drawings of a pair of living femora from a 6-day chick embryo one grown in normal and the other in +A medium During 9 days' cultivation the femur in normal medium elongated from 1 6 to 5 2 mm and showed no distortion That in +A medium grew normally for the first 2 days, then growth rapidly declined, a constriction appeared in the distal part of the shaft, and by the 9th day the condylar end was nearly detached from the diaphysis and the rudiment was shrunken and bent Reprinted, by permission, from Fell, H B, and Mellanby, E. The effect of hypervitaminosis A on embryonic limb-bones cultivated in vitro J Physiol 116, 320 (1952)

a highly developed thing in about ten days. Now, when this kind of material is planted in the hyper-A medium, a very different picture is obtained. In the first two days, there is no difference between the control and the hyper-A explant, but then the growth begins to fall off, and at the same time the consistency of the bone begins to change. Instead of being stiff and hard like the control, it becomes soft and gelatinous, and finally it actually begins to shrink.

Figure 11 shows the two series of camera lucida drawings of the two femora from a six-day embryo. That on the left is grown in normal medium, and that on the right in the hyper-A medium. You see that for the first two days there is practically no difference between the two. Then the growth rate begins to fall off as compared with the control. This becomes more pronounced, and, at nine days it has actually begun to diminish in size.

In some explants, especially in those of the humerus, a spontaneous fracture may occur and the head becomes completely detached from the shaft.

Travell: The head continues to grow, doesn't it?

Fell: It continues to grow and, as we shall see in a moment, ossification takes place quite actively in the plus A medium.

Fremont-Smith: On the vitamin A side, the head seemed to be growing more than the rest of it.

Fell: Yes, it is the shaft that seems to be particularly affected. Eventually, though, it is the whole explant. In sections of controls grown in normal medium, a layer of bone is seen to have developed in culture, while the cartilage has differentiated into a hypertrophic zone, flattened zones, and small-celled epiphyses as in the normal chick. The matrix stains very deeply with Delafield's hematoxylin. The corresponding rudiments grown in + A medium ossify, but although the cartilage cells differentiate, the cartilage matrix hardly stains at all. That is a very characteristic feature.

Fremont-Smith: Is there more calcification at that stage under the A than there is without it?

Fell: I don't think so, no. You will see a striking difference in the matrix in a preparation I shall show you in a minute, stained with toluidine blue.

Travell: Is the bone laid down along the shaft?

Fell: Yes, that is the cartilaginous model, and as in normal development the bone is laid down on the surface, where there is a spontaneous fracture, the periosteum may be disrupted and disorganized, and one obtains a diffuse ossification spreading out into



FIGURE 42 Ulna from a 6-day chick, embryo, grown in normal medium for 11 days Note the intense metachromatic coloration of the cartilage matrix. x 21 (Toluidine blue).

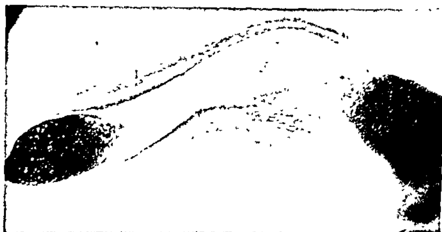


FIGURE 43 Ulna from the opposite side of the same embryo as in Figure 42, cultivated for 11 days in medium containing about 1200 IU of vitamin A per 100 ml Note the complete disappearance of the metachromasia from the shaft. x 21. (Toluidine blue)

the connective tissue Now, if you stain such preparations with toluidine blue, the difference in the reaction of the matrix is very obvious.

Figure 42 shows a control stained by toluidine blue. This is an ulna grown in normal medium for eleven days, and the intense metachromatic staining of the matrix may be seen.

Figure 43 shows the ulna from the opposite side of the same chick embryo after eleven days' cultivation in a medium containing about 1,200 IU of vitamin A per 100 ml. You see that the metachromasia has completely disappeared from the shaft.

Meyer. Does the hematoxylin stain the collagen?

Fell: We used Delafield's hematoxylin, which gives what appears to be almost a metachromatic stain with cartilage, as it does with mucus.

Meyer. So it stains the same material?

Fell: I think so, yes.

Meyer. The same material as the toluidine blue?

Dempsey. It probably does. It is an iron hematoxylin mixture and it probably stains acid materials but the chemistry of the hematoxylin stain is even less well known than the chemistry of the basic dyes.

Fell. I draw no conclusions about what chemical change is produced. *I merely record the fact that the staining reaction disappears* under the influence of vitamin A. It is a curious thing that different rudiments react in different degrees to the vitamin A. We have always found that the femur gives the earliest and most severe reaction, the humerus is next, then the tibia, and lastly the ulna and radius. You see the ulna in Figure 43 is quite a big rudiment and it shows the characteristic loss of metachromasia.

Dempsey. Is the staining in the two ends of the bone still present and is it also reduced in amount?

Fell. Eventually, most of it goes if you run it longer or if you have, perhaps, a rather *higher concentration*.

Dempsey. Most of it is in the shaft and it spreads.

Fell. It spreads, yes. Eventually one will be left with just a little blue patch at either end of the bone. It is rather curious, incidentally, how that compares with van Gieson's staining, because normally the cartilage scarcely takes up the red at all in van Gieson's stain. In these hyper A cultures the cartilage takes it up very strongly, *except where the metachromatic staining remains*.

Porter. That would mean then that the collagen was there but that the *matrix* of the cartilage had not been put down?

Fell. That is what we suspect.

Dempsey. Or a different matrix. I have been watching *for that*.

Fell. It actually prevents the matrix from forming, but it is also a loss. It becomes metachromatic and then the metachromasia disappears again.

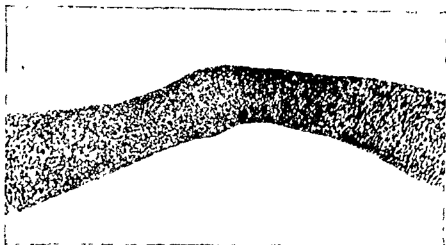


FIGURE 42 Ulna from a 6-day chick, embryo, grown in normal medium for 11 days Note the intense metachromatic coloration of the cartilage matrix x 21. (Toluidine blue).

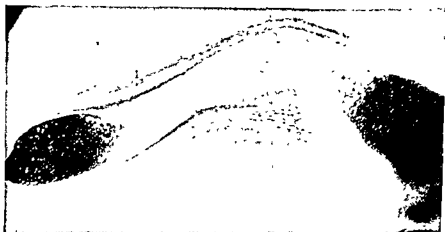


FIGURE 43 Ulna from the opposite side of the same embryo as in Figure 42, cultivated for 11 days in medium containing about 1200 IU of vitamin A per 100 ml Note the complete disappearance of the metachromasia from the shaft x 21 (Toluidine blue)

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Dempsey: Or a different matrix. I have been watching for that.

Fell: It actually prevents the matrix from forming, but it is also a loss. It becomes metachromatic and then the metachromasia disappears again.

Meyer: From the experiments with radioactive sulfate by Layton and Gardell, the turnover rate of sulfate in embryos seems to be very great Maybe some polysaccharide is desulfated without the sulfate group being removed, for example, by a process of transsulfation.

Dempsey Everything else may go except the sulfuric acid.

Meyer: From the experiments on the uptake of radioactive sulfur, we know that at this stage in embryos the turnover is really terrific. It may be that the sulfate groups are taken off but not put on again

Fell You mean that the turnover is expedited by the vitamin A?

Meyer: That would be very interesting. One might be able to determine total hexosamine, or hexuronic acid and sulfate, to find out whether vitamin A interferes with sulfate metabolism

Fell: That just indicates what we obtained with the very early, relatively undifferentiated skeletal rudiments. The effect on the well-developed bones was even more spectacular. For this work, we used the limb bones of late fetal mice, because they were so small, and of course when you want to grow *in vitro* a very hard, highly-developed structure, you have no vascular system, so you have to select something which is sufficiently small to survive with feeding entirely from the outside. In a normal limb bone of a mouse fetus near term, there is a well-developed bony shaft, a marrow cavity, and large cartilaginous ends. We used the radius, ulna and tibia from such fetuses They were of suitable size, and they remained, rather to our surprise, in a remarkably healthy state for as many as twelve days, which was as long as we had occasion to keep them.

Dempsey: Did you use the same medium, or is it a modified one?

Fell: We used the same medium; they did quite well on that.

Fremont-Smith You used the fowl?

Fell Yes

Dempsey: The fowl plasma?

Fell When we explant a bone of that sort in normal medium, it enlarges to some extent, but nothing like the rapidly growing chick rudiments As I say, it remains in a surprisingly healthy state for as long as twelve days. But the effect of the vitamin A on these explants was really quite spectacular. As before, there was almost no effect for the first two days and then suddenly, within twenty-four hours, the terminal cartilage began to shrink and the bone became rarefied. At the same time, the surrounding soft tissue grew very profusely, perhaps better than in the controls, and if growth continued long enough, eventually nothing remained in the hyper-A

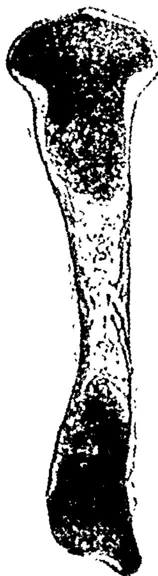


Figure 41. Tibia [illegible] *Brit. Med. J.* 2, 5[illegible] 19[illegible]

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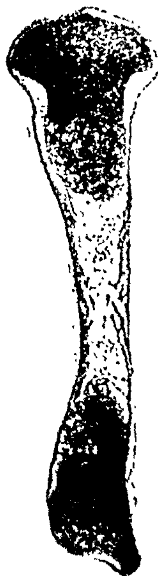
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[illegible]

medium but a sheet of ameboid cells, with a few crumbs of cartilage and bone lying about in it.

Figure 44 shows a control tibia grown in normal medium for seven days You can see the bony shaft, the marrow cavity, and the large basophilic cartilage ends, which have been enlarged and have become denser than they were at the time of explantation.

In Figure 45 we see the opposite tibia from the same fetus, after seven days' growth in a medium containing about 3,000 Inter-

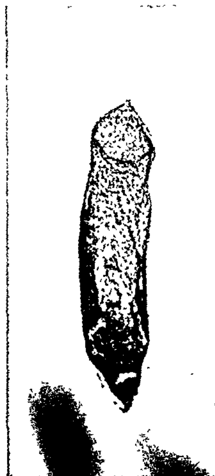


FIGURE 45 Tibia from the opposite side of the same fetus as in Figure 44, after 7 days' cultivation in medium containing about 3,000 IU of vitamin A per 100 ml. Very little cartilage or bone remains. x 25. Reprinted, by permission, from Fell, H. B., and Mellanby, E. Effects of hypervitaminosis A on foetal mouse bones cultivated in vitro, preliminary communication. Brit Med. J. 2, 535 (1950)

national units of vitamin A per 100 ml., which is not a very large amount as compared with what may be produced in the blood stream of an animal fed on a high A diet. You see how the cartilage has almost disappeared, this is a very characteristic effect. It shrank so that it is very much narrower than the bony shaft. It has a curious effect in life, like a little head and neck coming out of too large a collar. At this end, the cartilage is almost gone. These changes are not associated with necrosis, and the cells merely become more and more closely packed together as the matrix disappears. They quite often undergo mitotic cell division, and eventually all that is left is a compact mass of chondroblasts. If there is not too much extraneous tissue, eventually they seem simply to wander off into the culture medium and disappear altogether.

Figure 40 shows a high-power view of the cartilage from the hyper-A tibia grown in medium containing about 3,000 IU of vitamin A per 100 ml. You see, they are quite good cells, but all you have left of the matrix are these very thin capsules which now have lost their basophilia altogether. They stain with eosin at this stage, but otherwise there is nothing wrong with the cells. Then, as I said, the bones eventually disappear almost completely. For example, all that remains of a radius grown for ten days in medium containing 3,000 IU per 100 ml. is connective tissue, and often a little nodule of necrotic cartilage at the end. Such a little nodule is quite common in these explants, and it has a rather curious history. We traced it back to earlier stages and found that its origin was from the superficial cartilage at the articulation. When these little bones are disarticulated they are very toughly attached to each other, and the joint capsule is quite strong, but the articular cartilages are very fragile, and when the knife is inserted to separate them, the surface is usually slightly traumatized. The damaged cartilage in the controls is not noticeable unless it is very closely examined because it is a negligible part of the whole, but in these hyper-A cultures it becomes more and more obvious, because it is the only part of the structure that does not show the hyper-A changes. In general, the healthier the tissue and the more actively it is growing, the more profound is the effect of the vitamin A.

Porter: Dr. Fell, would you say that there is a decrease in total cell population?

Fell: I believe not. Of course, one loses a large amount of the cell population at transfer, because they just crawl out into the medium. I think if the normal amount of matrix were in between these cells (Figure 40), it would probably be found that the mass

medium but a sheet of ameboid cells, with a few crumbs of cartilage and bone lying about in it.

Figure 44 shows a control tibia grown in normal medium for seven days. You can see the bony shaft, the marrow cavity, and the large basophilic cartilage ends, which have been enlarged and have become denser than they were at the time of explantation.

In Figure 45 we see the opposite tibia from the same fetus, after seven days' growth in a medium containing about 3,000 Inter-

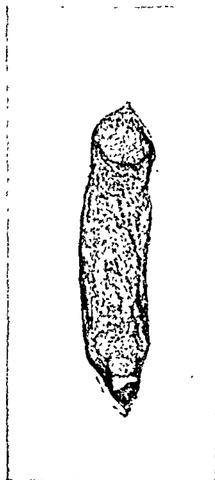


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Figure 46 shows a high-power view of the cartilage from the hyper-A tibia grown in medium containing about 3,000 IU of vitamin A per 100 ml. You see, they are quite good cells, but all you have left of the matrix are these very thin capsules which now have lost their basophilia altogether They stain with eosin at this stage, but otherwise there is nothing wrong with the cells Then, as I said, the bones eventually disappear almost completely. For example, all that remains of a radius grown for ten days in medium containing 3,000 IU per 100 ml is connective tissue, and often a little nodule of necrotic cartilage at the end Such a little nodule is quite common in these explants, and it has a rather curious history We traced it back to earlier stages and found that its origin was from the superficial cartilage at the articulation When these little bones are disarticulated they are very toughly attached to each other, and the joint capsule is quite strong, but the articular cartilages are very fragile, and when the knife is inserted to separate them, the surface is usually slightly traumatized. The damaged cartilage in the controls is not noticeable unless it is very closely examined because it is a negligible part of the whole, but in these hyper-A cultures it becomes more and more obvious, because it is the only part of the structure that does not show the hyper-A changes. In general, the healthier the tissue and the more actively it is growing, the more profound is the effect of the vitamin A

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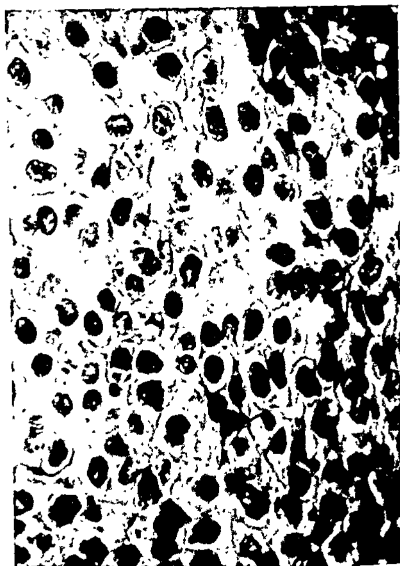


FIGURE 46 Area of terminal cartilage from the experimental tibia shown in Figure 45 Note the healthy appearance of the cartilage cells, which are close [illegible] cartilage matrix x 850

[illegible]per-

[illegible]tion.

was much the same. The diminution is at least due to the disappearance of the intercellular substance.

Travell. The little nodule of cartilage that remains represents an area of trauma?

Fell: Yes. And we have found in other experiments, too, that if you heat the bones to 45 degrees, for one-quarter hour, that prevents them from growing, and it also prevents any effect of the vitamin A appearing.

Travell. Are they viable?

Fell: No, they are not. Unless they are viable, the vitamin doesn't act. It acts through cell activity, apparently, and not directly on intercellular materials We have also found that a more pronounced effect is obtained with a moderate dose of vitamin A than with a very high dose, above a toxic level

Holbrook: Yet you just showed us one with 3,000 units that almost disappeared.

Fell: Well, 3,000 is well within the amount that may be obtained *in vivo* in the blood stream But if it is increased to about 8,000, there will not be a better, but a rather inferior effect

Holbrook: I understand.

Dempsey. So the removal of the cartilage matrix is a true metabolic effect?

Fell: Yes.

Dempsey: It is not a chemical exchange reaction that goes on?

Fell. No.

Dempsey: The vital activity of the cells participates in, and is necessary for, the removal of the matrix There isn't just a simple solubility of the material in the medium, by any means If a slice of fresh hyaline cartilage is placed in a dilute solution of toluidine blue, one may observe a streaming out from the cartilage of a material which turns the dye pink, and causes a metachromatic reaction in the surrounding dye That must be a very rapid affair, it must be a simple diffusion of soluble materials from the cartilage matrix into the dye That kind of effect, however, cannot explain these results that Dr Fell has been showing

Porter: No, but Dr Fell could test it by putting her explants in a maintenance medium, rather than a growth-stimulating medium, to see whether in the absence of vitamin A the amount of cartilage was reduced

Fell: Yes, I should think it might be, but that is only my guess But we do know, as I mentioned before that merely heating it to

45 degrees, which just prevents growth, also completely inhibits the vitamin A effect

Angevine. What happens to the cartilage that is stored in banks? Plastic surgeons use it quite frequently just as they do bone Have you any idea?

Fell: No, I am afraid I don't know. I have no information about it

Fremont-Smith But your work here should have a bearing on that.

Dempsey. The bank cartilage is kept in the frozen state, is it not, so it is in a state of suspended animation?

Angevine. I just mentioned it in relation to what you said, Dr. Porter, about the stability of the material.

Fell In all these experiments, we were working with what we called artificial hypervitaminosis, that is to say, we were taking normal plasma and adding to it vitamin A acetate, or alcohol We thought it would be interesting to find out how this effect compared with that which we might call the natural hypervitaminosis, i e, plasma containing excess vitamin A introduced into it by feeding the donor fowl Sir Edward put a number of fowl on a high A diet, then drew the plasma, estimated the amount of vitamin A present, and added the same quantity of the alcohol or the acetate to normal medium, and then we compared the effects. We found they were very different Qualitatively, they were the same With the natural hyper-A medium increased absorption of bone and cartilage, and a gradual loss of metachromasia are obtained, but the whole thing took place far more slowly than it did in controls grown in the artificial hyper-A medium I think this may be associated with the availability of the vitamin, because Sir Edward then made some extraction experiments with the two sets of plasma and found that in the artificial hyper-A all the vitamin could be extracted, with petrol ether, but that it could not be done with the natural hyper-A plasma unless the proteins were first denatured with 50 per cent alcohol Possibly the active bond is associated with the protein. I think there is some evidence for it in the literature.

Fremont-Smith. That is, protein in the plasma?

Fell: Yes

Porter. The animals, in a sense, are storing it or controlling the amount available?

Fell Yes Not only has the vitamin done something to the organism, but the organism has done something to the vitamin

Meyer: That means it is in coupled form, whatever that may be

Porter: Yes. The organism is attempting to regulate the amount of vitamin made available

Meyer: I should think it is the same problem. The transport form of that, by coupling, becomes more water-soluble.

Holbrook: There is evidently an attempt on the part of the animal to regulate its circulating vitamin A, it can store huge amounts of it in the liver for long periods, without altering its blood level

Meyer: Yes, that's right.

Fell. To summarize our results. it seemed quite clear that vitamin A, in concentrations in which it occurs in the blood stream of an animal, does act directly on skeletal tissue, and its action is not associated with cell degeneration It also seemed clear that the effect of the A was manifested mainly on the intercellular material, causing rapid shrinkage and the loss of metachromasia in the cartilage matrix and absorption of the bone The action of the vitamin *in vitro* was in many respects similar to its action in the young animal, but on the whole was more drastic Even in the case of the natural hypervitaminosis *in vitro*, it was more drastic and more rapid than in the young animal, and I think this effect is due to the fact that we were using younger tissue In general, we have found that the younger and more cellular the material, the more rapid and drastic is the effect of the vitamin, which again indicates that the vitamin is acting through cellular activity At present, we have no idea what the metabolic processes are which are involved in these changes

We were planning to undertake some biochemical investigations of this phenomenon and, in our simplicity, we thought that we might be able to restrict the field of our inquiry if we could produce another completely different effect of vitamin A *in vitro*, and so get a kind of cross-bearing on the problem It has been known for a very long time that in A deficiency, one of the most conspicuous effects is on certain mucous membranes, and Mori (6), and later Wolbach and Howe (7,8), have shown that stratified keratinizing epithelium replaces the normal epithelium in the respiratory tract, the genito-urinary tract, the salivary glands, and also in the nasal epithelium Sir Edward and I thought it would be interesting to see whether the converse effect could be obtained *in vitro*, that is to say, if the ectoderm were planted in a hyper-A medium, whether keratinization could be inhibited

It has long been known that when cultivated in normal medium, the *undifferentiated ectoderm develops into a stratified epithelium* and keratinizes quite profusely, so we prepared explants from the

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Fremont-Smith. That is, protein in the plasma?

Fell: Yes.

Porter. The animals, in a sense, are storing it or controlling the amount available?

Fell: Yes. Not only has the vitamin done something to the organism, but the organism has done something to the vitamin.

Meyer: That means it is in coupled form, whatever that may be.

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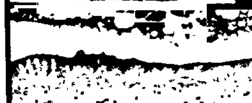
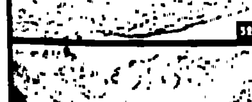
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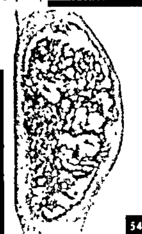
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FIGURE 50 Skin explant grown in +A medium for 14 days, watch glass *Note the* single layer of actively secreting columnar epithelium x 850 (Azan)

FIGURE 51 Control skin explant grown in normal medium for 11 days, part of an epithelial cyst in a hanging-drop culture The ectoderm has differentiated into squamous keratinizing epithelium x 380 (PAS, Mayer's hemalum).

FIGURE 52 Skin explant from the opposite side of the same embryo as that shown in Figure 51, after 11 days' growth in +A medium The ectoderm has differentiated into a secretory epithelium and the cavity of the cyst is filled with mucus x 380 (PAS, Mayer's hemalum)

FIGURE 53 Skin explant grown in +A medium for 11 days, hanging-drop culture *from the same experiment as for Figure 60, showing feather germ covered by* secretory epithelium x 180 (PAS, Mayer's hemalum)

FIGURE 54 Skin explant after 14 days' growth in +A medium, another section of the same hanging-drop culture as that shown in monochrome Figure 48 Note the secretory epithelium lining the cyst which is filled with mucus This epithelium is ciliated x 150 (PAS, Mayer's hemalum)

FIGURE 57 Normal nasal mucosa from the septum of an 18-day embryo, showing a mucous gland and ciliated cells x 470 (PAS, Mayer's hemalum).

FIGURE 59 Skin explant grown in +A medium for 7 days and then transferred to normal medium for 4 days, watch glass culture Note the squamous epithelium developing beneath the secretory, ciliated layer. x 600 (PAS, Mayer's hemalum).

FIGURE 60 Skin explant grown in +A medium for 11 days and then transferred to normal medium for 7 days, hanging-drop culture Note the squamous epithelium which is being formed beneath the goblet cells, the latter are bunched together and some are being shed x 250 (Azan)



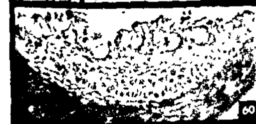
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one thing, and that was mucus; so I made sections of these explants, stained them by various stains that demonstrate mucus, and sure enough we had obtained the reverse condition of the deficiency *in vivo;* that is to say, we had a mucous metaplasia in response to the hypervitaminosis I shall show you one or two figures to illustrate that:

Figure 50 shows an azan preparation of a watch glass culture grown for fourteen days in plus-A medium The control, I should say, was completely keratinized at this stage, and was degenerate because it could no longer feed itself The columnar cells with the secretory vacuoles may be seen. Here is a sort of little glandular structure, such as appears quite commonly in the young nasal mucosa.

Bennett: Are they ciliated, too?

Fell. We shall come to that in a minute.

Fremont-Smith. This is surface epithelium, isn't it?

Fell: Yes; the seven-day ectoderm from the trunk or limb region – I don't know which – grown for fourteen days in a hyper-A medium

Figure 51 shows the control grown for eleven days in normal medium, in which the ectoderm has formed a complete cyst, this is just the wall of it.

In Figure 52 the opposite side of the same embryo appears grown in the hyper-A medium for eleven days Feather germs form quite readily, both in the normal and in the hyper-A ectoderm, but in the hyper-A *cultures they are covered, not by keratinized epithelium*, but by secretory epithelium

Dempsey. It seems to me that there is less collagen appearing in these hyper-A cultures than in the normal ones The basement membrane is less evident here than it was in the keratinized one Is that a correct impression?

Fell It could be, but the material was not sufficiently standard from that point of view for me to give a really critical answer In Figure 51 I should rather suspect the connective tissue to be affected, but I can not definitely answer that it is There isn't a great deal in that particular field

Dempsey: In one of the previous figures there was more Still, there is a line underneath the cell, and it gives the reticulum stain of this reaction

Fell: It certainly has an effect on cornea, which is what we are working on at the moment, so I suspect vitamin A does have an effect, however, I can not say that we have proved it

Porter. An interesting thing to me is that a cell which normally develops keratin intracellularly in the form of fine fibrils, now produces droplets of mucin, which are discharged from the cell surface.

Dempsey: I do not think that is surprising, though It occurs in certain of these epithelia, does it not? For example, in the vaginal mucosa of the rodent, there is, at one stage of the estrous cycle, a mucification of the surface epithelium, and in another stage they pile layer on layer and become cornified, so here the same cell layer goes through a physiological cycle of exactly this kind.

Fell. I have seen a number of pathological preparations of the human genital tract made by my colleague, Dr. Glucksmann, and some of the pictures are almost indistinguishable from what we obtained. The phenomenon, from the histological point of view, *seems very closely related.*

Dempsey: The paradoxical effect that Dr. Fell mentioned appears in the mucous epithelium of many of the mucous surfaces in hypervitaminosis in the entire organism, which transform themselves into stratified squamous keratinized epithelium.

Fell. In the deficiency.

Dempsey: Yes, I'm sorry; in the deficiency. I got it backwards.

Fremont-Smith: But this gives, at least to me, a further fillip to the idea of predestination, which depends upon the appropriate environment. When the environment is changed, the influence of heredity is also altered.

Dempsey. I think Dr Fremont-Smith believes in free will, to a limited degree

Travell: These cells couldn't change themselves into a muscle cell, or a neuron

Fremont-Smith: I agree, but we forget that all cells have the capacity to change, and what heredity will manifest at any time is always dependent upon the environment. I am going to run that in at every conference as long as I live. I think it is valid.

Travell. But these cells couldn't change themselves into mucous cells or neurons

Fremont-Smith: I don't want to predict negatively.

Dempsey: I wouldn't bet on it.

Fell: I'd bet a little, but no fortune.

Dempsey: Somebody is g another v e day.

Fremont-Smith: About all s that whe mani-fest normally, and in a pred ay, they lified But I do not say how far modific riate environ influences

Fell: Shall we go on to Figure 53. This is a feather germ The pigment cells developed quite well in the culture and they migrated up into the epithelium in the usual way, but they must have been rather surprised at what they found there.

Fremont-Smith: You see, the environment has changed

Dempsey: There is pigmented mucus.

Angevine: As development proceeds, is there a mucinous structure, or anything of that sort, as far as the surface of the chick is concerned. It doesn't have mucous glands, but does it have anything that is analogous? A frog does have mucous glands in the skin, and secretes mucus

Fell. Yes, and fish also

Angevine: Yes, fish have plenty of mucous glands

Holbrook. Dr. Fell, are you planning to discuss the effects on older tissue?

Fell: No, so far we have only worked on the younger tissue, which has taken all of our time

Fremont-Smith No senile chicks?

Fell. Not so far.

Porter: It would probably work the same way on older skin, since the skin in one sense remains an embryonic tissue

Fell. I don't know whether it will. That is something I have on my slate when I return.

Travell: Do you have a picture of a normal feather germ?

Fell: I am afraid I haven't They have the same appearance, with a layer of keratin over the surface, too

Fremont-Smith A thick layer of keratin instead of the mucous cells?

Travell. And pigment cells?

Fell. Yes

Porter: Is there any equivalent set of observations on the whole animal, when vitamin A is given?

Fremont-Smith: By using the egg, you mean?

Porter. Not necessarily the egg, but any animal after hyper doses

Fell A local effect has been described, but that was in mammals, which is not the same story We have done some preliminary experiments with mouse ectoderm, and so far we have not produced this effect. The ectoderm is either very much less sensitive to vitamin A in the mammal, or else the vitamin A doesn't get in I don't know which it is. We are hoping to do some fluorescent studies to see if we can find a clue to that point In mouse explants one needs a far higher dose to retard keratinization I have never com-

pletely inhibited it in the mouse, but hair formation may be inhibited. So far we have not obtained any metaplastic change.

Fremont-Smith: You haven't injected it into an egg and incubated the whole egg?

Fell: No, I haven't. Of course, it is a nice point as to how much of the vitamin will actually get into the chick. I don't know.

Fremont-Smith: It is an experiment that could be tried, is it not?

Fell: Yes; somebody did do some work on injecting vitamin A into eggs, but unfortunately did no histology on it, so one doesn't quite know what happened But it could be done.

Fremont-Smith: It would be lovely to see whether the chick could produce feathers.

Dempsey: Do you have comparable observations for the epithelium, when feather germs made in media that have been derived from high vitamin A donors are used?

Fell· No, we haven't. We should have, but we haven't

Dempsey: Is it possible that the increased effect in this case, as compared with the normal animal, is due to the conjugation of vitamin A; in other words, is it possible to obtain a higher concentration with free alcohol in the preparations?

Fell: I think that is very possible. I do not know whether it could be produced *in vivo* at all, or whether the bonding, or whatever it is that occurs with the use of vitamin A, would prevent this from happening.

Dempsey. The free vitamin in the blood just cannot go that high as long as the animal is alive. It is possible, I should think.

Fell. Yes, quite possible. This, of course, was a considerable shock to us, but one day I was looking through a set of hanging-drop cultures, just in a routine way, and I noticed a loose cell in one of the cysts spinning around like a top. I looked under a high power, and there I saw two ciliating cells in the epithelial wall which were responsible for the motion of the loose cell, and after that, I repeatedly got ciliary movement in these things. Sometimes there is a feather germ, and cilia beating near its base – a most fantastic picture.

Figure 54 shows a little cyst in a 14-day culture, full of mucus In Figure 55 we see another section of the same cyst, stained with azan, and the fringe of cilia. I can absolutely swear to these cilia because they were beating vigorously in life. My collaborator came up from London on purpose to see them

Porter: Is the ectoderm of the young embryo ciliated?

Fell· Not in the chick, but it seems to know how to do it.

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FIGURE 53. Skin explant grown in +A medium for 14 days, showing part of an epithelial vesicle, hanging-drop culture Note the cilia, which in life were seen actively beating Another section of this explant is shown in the color plate, Figure 51 x 950 (Azan) Reprinted, by permission, from Fell, H B, and Mellanby, E: Metaplasia produced in cultures of chick ectoderm by high vitamin A *J Physiol.* 119, 470 (1953)

Porter. It is reverting to an amphibian

Dempsey: Here is another kind of protein that these cells change over to.

Angevine: In tumors we occasionally see mucus in cells that are not normally concerned with the production of mucin In fibrocystic disease of the pancreas, in some way associated with vitamin A deficiency, there is an excessive secretion of mucin in certain ducts so that there is a tendency to call it mucinosis One sees mucin in small bile ducts in this disease, whereas one does not see it in these structures under normal conditions It would seem that there are conditions in man where somewhat analagous conditions may occur.

Fell Having produced these changes, we decided that the next obvious thing to do was to put the cultures back in normal medium and see what happened Here again we did not get exactly what



FIGURE 56 Skin explant grown in +A medium for 7 days and then transferred

we had expected The first result was that the secretory tissue already formed underwent a sudden spurt in development, and we obtained beautiful tracts of ciliated epithelium, and huge goblet cells The general histology was very similar to that of the normal nasal epithelium.

Figure 56 shows a watch glass culture that had been grown for seven days in plus-A medium, and four days in normal medium You see the whole surface is ciliated. This loose structure is quite characteristic, as the epithelium is very readily detached. But there is a layer of basal cells I should like to draw to your attention,

firmly attached to the connective tissue I shall have more to say about those in a moment.

Angevine: Dr. Fell, is there any alteration in polarity in these cells? Apparently, there was in the cartilage, and I just wondered if there would be in the epithelial cells

Fell: I don't quite understand your question

Angevine: Is there loss of polarity? In other words, is there normal orientation of one cell to another, that is, in relation to its neighbor? You would say "loss of normal organization," I assume

Fell. Meaning that they form these folds which seem very lightly stuck together?

Angevine: Yes, and is there an alteration in the normal relationship of one cell to another?

Fell. As compared with the epidermis, I think that is so, but what they, and the epithelium, most resemble, is the normal nasal epithelium

Figure 57 (page 161) shows the normal nasal mucosa of an 18-day chick embryo, and as you can see the general structure of the epithelium is very similar Here we have the cilia, and there is very much the same type of secretory structure

Travell. No basement membrane?

Meyer. That wouldn't be stained

Fell. It is not stained for that

Dempsey: What stain is used?

Fell: PAS (Periodic – Acid – Schiff)

Dempsey: How about your counterstain?

Fell The counterstain is Mayer's hemalum

Fremont-Smith: Aren't there experiments on transplants from one part of a growing embryo to another which show that cells which were normally predestined to form retina, or what not put in another part of the body will form the cells appropriate for that part of the body? We have quite a bit of evidence of that sort It might be that all cells of the body could become nasal mucous membrane cells if they were stimulated in the appropriate way

Fell: I think you would have to go back very early in development before determination had taken place

Fremont-Smith Yes, very early

Fell Much earlier than this It is astonishing, really that we get a metaplastic change as late as seven days, when all the organizers, you would suppose, had irrevocably acted

Fremont-Smith But the principle is the same I should like to challenge the idea of irrevocability

Holbrook: We are about to be told that all epithelia are potentially "nasaloid."

Dempsey: For the purpose of these conferences, which are for our self-education, what about the phenomenon of dedifferentiation? Is that in disrepute now? Is it possible for a cell, once it has differentiated, to revert to a more primitive condition and then redifferentiate in a different direction?

Fremont-Smith: This proves it isn't.

Dempsey: Not necessarily. There may be rests that have not yet differentiated.

Fell. But you have your basal cells there which are formative, as you say, and it is those which appear to be influenced.

For a hanging-drop preparation that has been grown in plus-A medium for eleven days, and in normal medium for seven days; there is a terrific outpouring of secretion I am astonished that under any circumstances, so much secretion can be obtained in a culture.

The next thing we noticed was that although the mucus-secreting tissue already formed had developed so well in normal medium, no new mucus-secreting cells differentiated, and instead the basal cells proliferated. The basal cells formed a squamous layer underneath the secretory cells, and eventually that keratinized and so reverted to the normal development of the trunk ectoderm.

Bennett: That is precisely what happens in the intact animal, isn't it?

Fell. It is. I had a delightful session with Dr. S B. Wolbach before I came here, and examined his figures of squamous metaplasia of mucous membranes in vitamin A deficiency. The histogenesis is identical with this recovery process It was most striking and very interesting.

Dempsey. You may be interested in another place where exactly this sequence is obtained: In the vaginal mucosa of the guinea pig, at estrus, you have a many-celled layer of keratinized, cornified epithelium, squamous on top, a basal layer below, and spinous cells in between After estrus, as soon as progesterone begins to act in the animal, the cornified layer is shed At about the third or fourth cell layer up from the basal cells it becomes mucified, and prior to mucification, these cells develop a considerable amount of glycogen The cell then turns into a mucous cell, and persists for a period of time until the next estrous cycle, when estrogen again stimulates the uterus, these cells are sloughed, and the layer corni-

fies from underneath and pushes up, so it is almost an exact parallel In this case it is maintained with a set of physiologically acting hormones rather than with the vitamins.

Fremont-Smith: Would it be sensible even to think about playing estrogen against progesterone in this kind of preparation?

Fell. I think it would, and that is one of the things we have in mind to do. One's progress is so slow



FIGURE 58 Skin explant grown for 11 days in +A medium and then transferred [illegible]

Figure 58 shows an azan preparation of a hanging-drop culture, grown for eleven days in hyper-A, and for four days in normal medium Here, you see the goblet cells with flattened basal nuclei, and here the basal layer is just beginning to acquire the squamous structure

Figure 59 (page 164) shows a watch glass culture, grown in plus-A medium for seven days, and normal medium for four days.

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FIGURE 58 Skin explant grown for 11 days in +A medium and then transferred to normal medium for 4 days, hanging-drop culture Note the typical goblet cells, distended with mucus and with flattened basal nuclei On the left of the photograph squamous epithelium is beginning to form beneath the goblet cells, a process which is well advanced in other parts of the vesicle x 700 (Azan) Reprinted by permission, from Fell, H B, and Mellanby E Metaplasia produced in cultures of chick ectoderm by high vitamin A *J Physiol* 119, 470 (1953)

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Figure 59 (page 164) shows a watch glass culture, grown in plus-A medium for seven days, and normal medium for four days

You can see a very nice little squamous epithelium sharply demarcated from the secretory ciliated layer, but you cannot see the cilia very clearly in this preparation. This figure is almost indistinguishable from some of Dr. Wolbach's preparations of squamous metaplasia in mucous membranes, in vitamin A-deficient animals (7). As these secretory cells are not replaced, in due course they degenerate and are shed

In Figure 60 (page 164) there is the beginning of that process. This preparation has been grown in plus-A medium for eleven days, and in normal medium for seven days, and here the secretory cells are being bunched together, beginning to get necrotic. You can see the squamous epithelium developing underneath



Figure 61 shows the last stage. Here is a mass of keratin, squamous epithelium; a few mucus-secreting cells are left, and there is still some mucus in the cavity This recovery process takes place

at different rates in different parts of the culture It can usually be found in all stages in the same explant

Holbrook: I should like to ask Dr Fell if, having shown us what can be done with a nonsecretory cell to make it secretory, she is going to talk to us about what can be done with a primary secretory cell in relation to its secretion?

Fell· Yes, I have some information about that Of course the interpretation of these results is extremely difficult, but certain things seem to emerge I think our results serve to emphasize what has already been shown in animals, that vitamin A is an essential factor in the control of keratinization. It seems that in the intact animal, a certain amount of the vitamin is required to suppress keratinization, and that the minimum level varies very widely for different epithelia Thus the level normally present in the blood is sufficient to suppress keratinization for example, in the normal nasal epithelium, but if the concentration falls below the level as in A deficiency, then keratinization takes place

Now with these chick ectoderm cultures, the same principle seems to hold, except that the threshold is very much higher If there is a very high level of vitamin A, the squamous differentiation and the keratinization may be suppressed, but if the level falls to that normally present in the body, then keratinization takes place Of course, we are faced with this extraordinary metaplastic change in the cultures. One thing we can say about it is that the vitamin seems to act entirely on the basal cells, and then too it is a qualitative effect, that is to say, it causes the transformation of the basal cells into the mucous epithelium, but we have no evidence that it affects mucus secretion. Recently, we have done a number of experiments in which we have made organ cultures of various secretory tissues, such as the nasal epithelium and gut, and they secrete profusely both in the normal and in the hyper-A medium Personally I can see no difference whatever between the two explants in the two media There is no evidence that vitamin A promotes secretion in what is normally a secretory tissue, and I believe that that is the experience of people who work with hyperavitaminosis in animals

Porter: I wonder what keratin and the mucin involved here have in common?

Meyer: I don't know of any similarity It seems to me that the explanation could lie in whether the manufacturing plant of the cell is determined by the amount of vitamin A It has potentially the system either to produce keratin, or cilia and mucin If it

is under the influence of an oversupply of vitamin A, the keratin-producing facility is switched over to that of a mucin-producing facility, and once that is established that cell cannot be changed, so presumably it ought to influence the suppression of the genetically present potential of the cell

Fell: We have no evidence that when a cell is differentiated – for example, for mucus secretion – it can then be transformed into a keratinizing cell It just degenerates.

Dempsey: One thing both substances have in common is a high sulfur content, but in a very different form. One has a sulfate and the other has a sulfhydryl disulfide. As a rule, mucins produced by ectodermal structures do not contain sulfate. The only exception I know is the mucoitin sulfate produced by some cells of the gastric mucosa (9). It has been shown by a number of workers that this sulfated mucopolysaccharide occurs in the gastric secretion.

Porter: The polysaccharide component is the thing that is being produced here in the mucin cells, and not in the keratin cells.

Meyer. Yes, though I doubt it contains sulfate.

Dempsey: How about the mucus of the cervix? Does it not have sulfuric acid in it?

Meyer: Cervical mucus has been studied by Shettles, Dische and Osnos (10) and also by Werner (9) with colorimetric methods. They found blood group mucoids in this secretion The carbohydrate moiety apparently contained hexosamine, galactose, fucose and, according to Werner, a mucoid called sialic acid, but no sulfate.

Dempsey. Histologically that doesn't fit very well, Dr. Meyer. The epithelial mucus found in goblet cells in the surface epithelia of lower animals, in the nasal mucus, in the uterine cervix, and in other places, is an extraordinarily basophilic substance, perhaps the strongest basophilic material anywhere in the body. On the other hand, the mucus of the gastrointestinal tract, and of the stomach mucosa, in which neutral polysaccharides have been identified, is scarcely basophilic at all, which it should not be. It is also perhaps one of the most strongly metachromatic substances to be found anywhere in the body· as strong or stronger than cartilage.

Meyer: There is a strongly sulfated polysaccharide in the mucus and in the mucosa.

Dempsey: Nevertheless, there are mucous cells which are not basophilic. They are both there, which is my point, so I should certainly suspect that these things are strongly acid, whether or not they contain a sulfuric acid group. I thought the uterine cervical mucus had been found to contain sulfuric acid, but I do not know.

Meyer: The work I quoted is on human mucus I do not know of any other work. I do not believe I should dare to make any prediction as to what the nature of this material is in the lower species, or in the vaginal mucus Did you test it Dr. Fell? Was it metachromatic?

Fell: Yes, it was metachromatic with toluidine blue

Meyer: Strongly metachromatic?

Fell: No, not very, I think, but I speak very doubtfully about it because I have not properly gone into the matter

Porter: Dr. Fell, I think it might be pointed out, too, that mucin is not seen in the ciliated cells, it is rather in the adjacent cells This development of ciliated cells may be considered a secondary response to the presence of a mucin-forming membrane

Dempsey: At one point you asked whether or not the mucous cells that had little whiskers on the top were ciliary

Fell: Yes, I thought there were in normal nasal mucosa

Porter: I don't think so

Dempsey: They were side by side I think Dr Porter is right You find a ciliary cell and a mucous cell next to it, but never the two sharing the same cell

Bennett. The ciliated cell appeared later in your cultures

Fremont-Smith: When you observed it in life did you get evidence of mucus secretion by ciliated cells?

Fell. No, I can't say from life. I certainly know that by far the greater part of the mucus secretion is not in the ciliated cells That I am quite certain about But I am not quite so sure whether the ciliated cells secrete nothing.

Porter. I think not.

Fell: You may be right, I am not sure. They have a good deal of gadgetry inside. I dare say there is a lot of PAS-positive material in the ciliated cells which is not mucus

Dempsey. The Fallopian tube is ciliated, but not any organs found lower than that in the body.

Fell: The esophagus has a transitory ciliated stage there is a very nice paper about that.

Dempsey: I should like to avoid this argument about whether or not the mucus contains sulfate, but I should still like to come back to the problem and ask Dr Meyer if there is any biochemical mechanism he knows of whereby a sulfur-containing precursor might be diverted by one set of machinery into forming something

like keratin, and by another set of machinery into forming a sulfated mucopolysaccharide. Is it conceivable that a single precursor would come to the cells and might manage to . . .

Meyer: The origin of ester sulfate apparently is inorganic sulfate, *while that of the SH and* SCH_3 *groups is organic dietary sulfur.*

Dempsey: But isn't the converse also true, that the SH and SS which we ingest may be oxidized to sulfate and excreted as sulfate?

Meyer. I think most of the cystine-S is excreted as sulfonic acid in taurine. I think the C-S bond in sulfonic acid is split only to a slight degree

Dempsey: There is sulfate, though, that is excreted in the urine?

Meyer: Oh, yes, there is plenty of it.

Dempsey: Now, is the origin of that sulfate exclusively ingested sulfate?

Meyer: No, the origin of excreted sulfate is partly derived from oxidation of organic sulfur.

Dempsey: So it is possible, it seems to me, for a sulfur-containing precursor to be routed, by these cells, from either one of these directions?

Meyer: I should still assume that the amino acid composition of the keratin ought to be quite different from that of these mucins, but we do not know the nature of it

Porter: Maybe the protein portion is quite similar.

Meyer That is possible

Fell: Looking at it as an embryologist, I couldn't help but wonder whether all the vitamin A did was to suppress the keratinization

Porter. To suppress one set of plasmagenes and encourage another?

Fell· Yes; the ectoderm pursues a route which, in ordinary development, it follows in the nasal region. That is just a guess, though

Dempsey. Isn't there another set of experimental conditions in which the same kind of change can occur? For example, in tracheotomy, does the tracheal epithelium frequently keratinize around the exposed edge of the wound?

Bennett That happens in situations where there is long continued irritation, but where there is no evidence to suggest the presence of vitamin A deficiency. Recovery takes place after the irritative phenomena have been removed. We do not know whether there is interference with the utilization of vitamin A locally or not, but we do see this metaplastic change in association with numerous pathologic situations.

Porter: There is possibly a space relationship there, too The mucus-forming cells are not as far removed from the dermis as the keratin-forming cells, are they?

Dempsey. No, and there is another space relationship which is one I was trying to get to These epithelia are oriented, on the surface of the body, with respect to air on one side, and interstitial fluid and the blood stream on the other. At the base of the cell, there is an intercellular environment dominated by the blood stream and tissue fluids At the surface, there is an environment which is dominated by atmospheric oxygen. In between one has all transitions. In the tracheotomy experiment which I mentioned, and in many other pathological situations when a mucous membrane is exposed to dry air, it keratinizes If the air is made moist again, as it normally is in the trachea, it is mucified If one alters the relations between atmospheric oxygen, moisture, and the oxidative mechanisms of the blood stream of the ordinary growing cell isolated from there, one may obtain any transition It seems to me that somewhere among these parameters there is the mechanism which induces these changes

Meyer. Isn't the corneal type of epithelium, especially. one which is keratinized very readily if exposed to the air, for example, if it is dried out?

Dempsey: Yes.

Meyer. The cornea keratinizes very readily in vitamin A deficiency and in many pathological conditions

Fremont-Smith: But vitamin A deficiency would accentuate it, wouldn't it?

Meyer: It should accentuate the keratinization. and this is not a matter of fluid. I do not believe there is anything known about the tear gland secretion dropping in vitamin A deficiency Does it do so?

Fell: I have no idea

Holbrook: There has been some discussion about that Dr Meyer There is a syndrome known as Sjogren's syndrome. in which the eyes are dry and there is a dry mucous membrane

Meyer. Conjunctivitis sicca.

Holbrook: That's right, which is one phase of the syndrome It is not too common a condition, but curiously enough, there have been some reports of those individuals having vitamin A deficiency I have tried modest amounts of vitamin A with them, without any improvement at all.

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Meyer In this disease the conjunctival epithelium is partly replaced by a mass of goblet cells.

Angevine It would be rather interesting, in view of the discussion here, to examine some of the organs from X disease in cattle, which is apparently associated with destruction of the ability to utilize vitamin A There is keratinization of the nose in affected animals, and there is a high mortality among them. If one could get some of their offspring, perhaps very early, they might be a very interesting group to look at from this standpoint.

Holbrook. The calves die if the cow has the disease.

Angevine· Therefore, it should be possible to obtain a calf.

Fremont-Smith· The embryos might be interesting.

Angevine Yes.

Fremont-Smith What is the name of the disease?

Angevine: X disease in cattle.

Fremont-Smith. "X" meaning unknown?

Angevine Yes. They thought for a long time it was a virus, but they can maintain these cattle fairly well by giving them large doses of vitamin A. They cannot reverse the process once it gets started

Holbrook: The calves will live if the mother is given vitamin A

Fell· There is nothing known about the pathology of it?

Holbrook· Nothing.

Anvegine. I wouldn't say that, I don't know.

Fremont-Smith· But the embryonic pathology has not been determined?

Angevine No, it has never been touched so far as I know. I shall try to obtain tissues to see what we can find. I should have an excellent source of material for it.

Fell Is it possible that in some conditions the individual is not suffering from A deficiency but, for some reason, an inability of the tissues to metabolize vitamin A?

Angevine Yes, I think so.

Travell Dr Dempsey spoke about changes in the environment at the surface of the skin: the amount of moisture, and the amount of oxygen dominating What effect do these amounts of vitamin A have on the oxidative processes of the explant?

Fell· I have no idea.

Holbrook: Dr Fell, you told us that this didn't work so well in mammals, and yet you showed us a young rat that had spontaneous fractures

Fell. Oh, yes. It works extremely well on the skeleton of mammals, but we haven't got the metaplastic change in mammalian skin.

Holbrook: Then my next question is, have you any information about what happens in the skeleton of old rats, with the same or larger doses of vitamin A?

Fell. I believe not very much. It is a change peculiar to the growing skeleton. That ties in with our findings that the younger, the more cellular the tissue, the more pronounced is the effect. We did some experiments with newborn rat or mouse bones and the effect was very much slower, and less drastic, than it was with the younger fetuses.

Dempsey: There is an argument that one is dealing here with a change in sulfur metabolism, which, I think, can be extended to the observations you made on cartilage matrix, isn't that so? One starts out with a situation in which there is plasma as a nutrient material, and from which the explant normally synthesizes cartilage matrix, which contains sulfate. Now, in the hypervitaminosis A, the explant cannot synthesize the sulfur-containing matrix and indeed it loses what it already has, and so somewhere there is a block in the formation of the sulfate that goes into the cartilage matrix from the precursors of the plasma, just as we seem to have a similar mechanism turning up in the epithelium.

May I ask a general question, does hypervitaminosis A produce any changes in places like the chief cells of the gastric mucosa or in places where one obtains mucoid materials other than the sulfated ones? In other words, I wonder if there is any alteration in the formation of any of the neutral polysaccharides?

Bennett: In so far as I know, the only places where these metaplastic changes occur are the sites referred to by Dr Fell: the respiratory tract including the bronchi, the urinary tract including the renal pelvis, the ureters and bladder. I do not know of any places along the gastrointestinal tract where the lesion occurs. Do you know of any, Dr. Angevine?

Angevine: No.

Fell. I believe it is singularly free from the effects for reasons which are quite unknown. It is probably a very significant difference.

Bennett: The uterus, of course, is very profoundly affected.

Dempsey: The snail would be a good animal to use. It leaves a track of mucus behind it.

Holbrook: Are there any further questions or discussion? It was a very interesting story, Dr. Fell, I think you ought to tell us what you propose to do next.

Meyer: In this disease the conjunctival epithelium is partly replaced by a mass of goblet cells

Angevine: It would be rather interesting, in view of the discussion here, to examine some of the organs from X disease in cattle, which is apparently associated with destruction of the ability to utilize vitamin A. There is keratinization of the nose in affected animals, and there is a high mortality among them. If one could get some of their offspring, perhaps very early, they might be a very interesting group to look at from this standpoint.

Holbrook: The calves die if the cow has the disease.

Angevine: Therefore, it should be possible to obtain a calf.

Fremont-Smith: The embryos might be interesting.

Angevine Yes.

Fremont-Smith. What is the name of the disease?

Angevine· X disease in cattle

Fremont-Smith: "X" meaning unknown?

Angevine Yes They thought for a long time it was a virus, but they can maintain these cattle fairly well by giving them large doses of vitamin A. They cannot reverse the process once it gets started

Holbrook The calves will live if the mother is given vitamin A.

Fell. There is nothing known about the pathology of it?

Holbrook. Nothing

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Holbrook. Are there any further questions or discussion? It was a very interesting story, Dr. Fell, I think you ought to tell us what you propose to do next.

Fell: There are many things we propose to do. We should like to try the effects on older epithelium, and also go properly into the mammalian question to see whether there is a difference, and whether the mucous change can be produced with varying conditions I should also like to know whether the vitamin gets into the epithelium of the mammal, and if so, whether that can be the explanation for the difference. I do not know. It has been demonstrated, I believe, by fluorescence in epithelia.

Holbrook Have you used the purer synthetic vitamin A at all in any of these recent studies?

Fell. We used the purest we could obtain — the crystalline acetate I don't suppose any of them are really pure, are they?

Meyer. There are now different isomers of vitamin A. I do not believe the mechanisms of the mode of action of any of the fat soluble vitamins is known. They must somehow influence the metabolism of the cells, but whether this happens inside the cell, or on the surface, I don't think is known.

Porter. Dr. Fell, do you think the histogenesis, or the cytogenesis, of this mucin followed the normal pattern of mucin formation?

Fell: There are a good many granules in the cell, but I am afraid I haven't gone into them cytologically in any great detail.

Fremont-Smith. Dr. Fell, have you used other organs of the body for explants, such as the uterus, as well as tissues of the epithelium, bone, and connective tissue?

Fell No, not yet

Fremont-Smith· That is possible, isn't it?

Fell· It is all planned, yes, and we are going to try a number of tissues when I return.

Fremont-Smith: What about the use of the egg as a place in which to incubate the mammal or the mammalian tissues? That has been done, I think Have you tried that?

Fell. No, but it can be used

Fremont-Smith It has been done by someone who was working with Dr Gunn on the embryo mouse. I was wondering whether there were advantages and disadvantages

Fell. What we were after was this direct effect of A It can only be demonstrated under the simplest of conditions. Once the vitamin is put in the body, you do not really know what happens to it, as was illustrated by the comparison between the natural and the artificial hyperplasia Not only does the agent affect the animal, but the animal alters the agent. I think that is one of the advantages

of the organ culture method because it does enable one to isolate certain bits of a problem and so use it as an adjunct to experiments *in vivo, though practically never as a substitute for them.*

Porter: This is the first demonstration, is it not, that the vitamin can act directly on the cell or tissue without being mediated by the whole animal?

Fell. It is the first evidence that it can in concentrations in which it occurs *in vivo* There were some very nice experiments by Barnicot (11) in which he put crystals of vitamin A acetate on bone grafts and got resorption, which was pretty good evidence of the direct action But then, of course, that was a terrific concentration

Dempsey. You haven't tried to grow eyes or retina, have you? This is another place, of course, where vitamin A goes directly into the structural part of the cell, and it might be very interesting

Fell. No, that is a thing we are planning to do The retina develops extremely nicely in culture

Fremont-Smith: It does?

Fell. Yes One starts with a three-day eye, and almost an adult retina is obtained in an organ culture The same is true of the otocyst, and we are going to try both experiments when I return and see what effect, if any, A has on them

Dempsey: That ought to be fascinating

Meyer: You did some work with cornea?

Fell: Yes, we have done some preliminary experiments on that, but I cannot tell you much about them

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OUTLINE OF PROBLEMS TO BE SOLVED IN THE STUDY OF CONNECTIVE TISSUES

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This outline will be subject to the limitations imposed by the extent of the information available at the present time on this and related subjects, and by the bias of one man who will try to put it together. For the purpose of dialectics, that is, in order to analyze and systematize what we are trying to do, I have divided the problem into two topics: the analysis of structure and of function Obviously a division is artificial because there are spheres where the two overlap, both in method and in concept However, it seemed to me that this division would be useful in clarifying the concepts which we have, or are trying to form The analysis of structure involves the physical aspect, that is, histology and the fine structure as well as the chemical composition of the substances and their nature. The functional aspect is what Dr Ragan calls "dynamics," and this term could probably be substituted for the term "analysis of function"

In making this outline, I thought first of the histological and histochemical methods available at the present time However, I know very little about that subject, so I shall just intimate that I think it is one of the most fruitful areas of endeavor if the methods could be improved For example, in the field of ground substances, with which I am perhaps most familiar, we find in many tissues three different mucopolysaccharides some loosely bound, and others intimately bound, to proteins The histologist tells us that he finds metachromasia present or absent in certain localizations and under certain circumstances It seems to me imperative that the histologist find out what the exact localization is of the substances which the chemist extracts from the mixed tissues and characterizes after fractionation and analysis

I should be very grateful for any suggestions as to how one might discover whether these fractions occur in the tissues as random

mixtures — which personally I do not believe — or whether they are localized as discrete structures The *Kittsubstanz* of the old German literature, versus the *Grundsubstanz* obviously indicated a duality of structures which may correspond to the two (or three) mucopolysaccharides or mucoproteins isolated from connective tissue How to approach the histological problem, I do not know. The unfortunate thing is that the carbohydrates lack distinguishing features except in the infrared side of the spectrum Whether or not infrared microspectroscopy is possible, I do not know.

Porter: Something might be done with electron microscopy.

Meyer. The trouble with electron microscopy is that the substances are not highly organized. If enzymatic removal is used, one must have specific enzymes. Histologists have been using such methods for many years The enzymes which are available now are not as specific as they were thought to be In the test tube, chondroitin sulfate B is resistant to the action of testicular and bacterial hyaluronidase Yet it is reported that the metachromasia of skin, for example, does disappear completely after incubation with testicular hyaluronidase. The same has been reported for the granules of mast cells. Heparin, which is contained in the granules, is completely resistant to hyaluronidase action. Yet the metachromasia of the granules is reported to disappear after incubation with testicular hyaluronidase.

The difficulty is that the enzymes used may be quite impure. Recently, for example, I saw a note, I believe in the *Proceedings of the Biochemical Society*, in which somebody investigated the protease content of a variety of hyaluronidase preparations All had proteolytic activity, and in fact it increased with the increase in hyaluronidase activity I am sure the two enzymes have nothing in common. One might suspect that some effects on ground substances described in the literature may be due to this proteolytic activity Day, in England, has shown that proteolytic enzymes are very effective in removing the ground substances in loose connective tissue.

Fell. Couldn't one do the experiments with protease inhibitor?

Meyer· Yes, that could be done. Perhaps Dr. Hass ought to think of using a trypsin inhibitor in his experiments. The trouble with the inhibitors is that they have definite specificities The trypsin inhibitors of eggwhite, soy beans, or that found in the pancreas, may not act on a tissue protease. Also, the concentration of these substances per unit area, or unit volume, are probably very small, and

one is faced with the problem of the limits of the resolution by histological methods

Porter. Of course, one cannot decide these things very well unless one tries them, but I think that electron microscopy does offer one type of approach to the problem of localization, which has not, up to this time, been developed at all. There must be many reagents that one could use in electron microscopy which would be bound rather specifically by the reactive groups in the ground substance, and they could thus be localized

Fell: If there were a protease acting, wouldn't you see signs of its activity in other ways?

Meyer: I do not know. One might expect that the tissue proteases would hydrolyze the proteins of the ground substances, while the structural elements might not be attacked at all, or only very slowly, except by collagenase, the chemical specificity of which, I believe, is not known

The next item in my outline is the chemistry of the connective tissues There, with known procedures, a lot more can be learned than we know at present. For example, we have just finished some work on cornea, and isolated three distinct acid mucopolysaccharides from this tissue, among them a novel type composed of N-acetylglucosamine, galactose, and sulfate, which we had overlooked, or rather lost, in the fractionation procedure which we used over ten years ago Now we have reason to suspect that such a polysaccharide occurs also in tissues other than the cornea, although it was not detected in pigskin or in tendon The two other polysaccharides which accompany this one are a chondroitin sulfate, and a fraction which looks like hyaluronic acid, although the rotation of the fraction is not the same as in hyaluronic acid from other sources The separation of these fractions is not easy, but it can be done, and I believe the chemical structure of these compounds can be elucidated. This, I feel, is still a very fruitful field For example, as discussed earlier, the effect of hypervitaminosis A on cartilage could be studied by experimental methods This could be done by a combination of chemical and histological techniques It is quite obvious that guidance should be provided by the histologist, as the [illegible], and it would b[illegible] predict what w[illegible]ing from one step to the next, to find out what happens under the guidance of histology.

The analysis of function, I personally believe, is almost com-

pletely *terra incognita* We have nothing to go on, at present, but *it is quite obvious from reading the literature, and from pure* deductive reasoning, that it ought to be one of the most fascinating fields If we wish to progress in our understanding of pathology, the only rational approach is through the mechanisms, and I have subdivided these very artificially into physiological, pathological, and pharmacological, under which I have listed growth and development, aging, calcification, salt and water metabolism, energy metabolism *and its relation to specific functions — as previously* discussed — and the interrelations of structure with chemical reactions.

Under pathology, one could make a list of several pages, listing in detail the studies which could be made. The same applies to pharmacology, under which I placed all the actions or reactions to hormones, toxins, and other exogenous products, and left it rather undetermined, then, the reactions to drugs, and, as the last point, which I think we have completely lost track of, the interaction with parenchymatous organs and with the organism as a whole When Dr Fell showed us those beautiful pictures my first reaction was. *this is again action on some connective tissue elements* rather than on the epithelium. The epithelium is indirectly affected because it depends for its function, and part of its metabolism, on what it obtains from the stroma. If the stroma does not function properly, the epithelium also will not function This is a completely unexplored area.

I should like to conclude with the statement that the outline obviously *includes almost every problem of biology* and medicine. This should not be surprising, in view of the very simple fact that we have connective tissue everywhere, and that it is involved in *all processes with which we come* in contact, either in physiology or in pathology

Dempsey· From what Dr Meyer has said, I feel there is a distinction perhaps, between histological methods, on the one hand, and chemical on the other He seems to feel that the known histological methods have been applied more widely than have the known chemical methods. Consequently, work with histological methods will probably require the development of new techniques, whereas the way is a little bit clearer in chemistry, where the techniques are better known Was that what you meant, Dr. Meyer?

Meyer Yes, I think in chemistry the paths by which we should and could go are much more clearly outlined than in histology.

Dempsey· The field has been pretty well plowed in existing histological methods One exception to that is probably electron

microscopy, which has not been so widely applied to these tissues as it could be.

Meyer. That is probably true I don't know enough about the x-ray spectroscopy of – what is the name of the Swedish worker?

Dempsey. Engstrom. That is really not histology though, it seems to me it is almost another field altogether.

Meyer: To me, it is histology, namely, the localization and the quantity of substances present in a given area

Angevine. With an x-ray spectroscope one can measure the mass of material in a cell One can also remove part of it, or combine it with something and determine the mass before or after such a procedure One is limited by (*a*) magnification, (*b*) grain of film, and (*c*) the quantity of material present. It is being used in a few places only, but I think eventually it will be a useful tool At present it is necessary to develop accurate techniques to be used in association with the application of the instrument We have under construction at present an x-ray spectrograph which, if a success, will materially enhance the value of this technique

Meyer: Couldn't one combine, let us say, the reactive groups with specially heavy, opaque, dense atoms or molecules?

Angevine: Yes.

Meyer. One could do the same with a heavy metal which has some very special affinity for certain acidic groups, or it could be done with basic groups.

Dempsey: But isn't that essentially the same phenomenon that one deals with in electron microscopy?

Porter: Yes, it is.

Dempsey: X-ray density, electron diffraction and scattering power are essentially the same thing, are they not?

Holbrook: What one really needs is a machine like the one Dr Hass has, to get several hundred grams of ground substance together as he did with his fibrils

Meyer: No, for chemistry, you don't.

Dempsey: Not for chemistry, but we shall wish to know where each one of these fibrils comes from

Holbrook: For orientation, certainly

Travell. Ground substance isn't enclosed in a nice capsule like the myofibril.

Holbrook: That adds to the problem.

Meyer. Van den Hooff (1) has a figure in his paper on the cornea, in which he shows that the ground substances in the cornea may be seen as sleeves around the collagenous fibrils.

Porter: I haven't seen the paper, so I do not know. I think we shall find that quite a lot can be done with frozen-dried material in the electron microscope.

Dempsey: Dr Porter, don't you think we need to develop a series of what you might call histochemical methods for the electron microscope: a series of methods designed to characterize in chemical terms the substances that may be seen with the electron microscope, just as is being done with the light microscope? Such a technique is practically nonexistent at the present time.

Porter: That's right; but we shall have it eventually.

Dempsey: Yes, but it comes again into Dr. Meyer's category of things that must await the development of method. There are a great many more histochemical methods that may be developed by improvement of existing methods

Meyer. I am rather optimistic.

Porter: How many enzymes will diffuse through collodion, when used as an imbedding matrix?

Meyer: There are enzymes which pass a collodion or cellophane membrane, that is, those which have a molecular weight of 15,000 or less. The permeability of collodion membranes depends upon their preparation

Dempsey You really mean methacrylate membranes, don't you?

Porter: No, you can imbed them in collodion.

Meyer I would not rely on diffusion into collodion.

Dempsey: I should like to recommend a project that could be done in Dr. Porter's laboratory. What we need in this field for beginning histochemical work with the electron microscope is a means of fixing tissue so that sections may be cut without mechanical or other distortion of the tissue, but which does not involve the introduction of an electron-dense material; in other words what we need is a fixative that is as good as osmic acid, and which does not have the electron-scattering power of osmium.

Porter I think probably formalin, if properly used, would be almost as good; it meets those requirements

Meyer. Except that some of these polysaccharides are really leached out, and certainly they are diffused from one place to the next.

Porter: Formaldehyde will not fix lipids, but the protein framework, if such it may be called, can be preserved with it quite well

Dempsey. Freeze-drying, and its improvements, are other possibilities.

Holbrook: What about the chemical structure of the ground

substance, Dr. Meyer? Are methods available to tell us, with some reasonable accuracy, what amino acids are utilized?

Meyer. Do you mean the protein complex?

Holbrook: Yes, which protein fraction?

Meyer There are methods for analysis. Naturally, the first step would have to be the isolation, in so-called pure form, of the substances. For example, in cartilage one thing has always been a riddle to me. with what protein are the two chondroitin sulfates which we can recognize combined?

Holbrook: That is what I am asking.

Meyer: Is this collagen? I know that the electron microscopists show us beautiful collagen fibrils in cartilage, but in that area, in the zones which are so strongly metachromatic, what is the protein? And what is the physical or chemical relationship between these carbohydrates and the protein present there? There is protein, by the way If it is collagen — and I have always stressed the "if" — it certainly has a widely different solubility from the collagen of the skin, for example, or of tendon.

Fell: In electron microscopic preparations of young cartilage — young chick cartilage, for example — very little collagen is found One has to look very carefully to find a few fibers, but one does find a mass of fine fibered material What that is, I don't know The Kings College people have been seeing it It has apparently no periodic structure.

Meyer. Is the ground substance just metachromatic?

Fell Yes, at a stage when the ordinary material is strongly metachromatic.

Meyer. This is hyaline cartilage, obviously

In answer to your question, Dr. Holbrook, it happens that the hyaline cartilage is one of the cleanest tissues to work with, compared with loose connective tissue. Nobody has done anything to my knowledge, about the problem The same applies, I suppose, to the cornea, where one may have a rather clean connective tissue if one removes the epithelium and the endothelium by a very simple method

Holbrook: Nothing is known at the present time about the composition of the protein?

Meyer: No, except that in tendon and heart valves we did find, [illegible] cal with

[illegible] Meyer?

Meyer: Of course bias, personal experience, and so on, enters

into it So far as the availability of the method goes, I should say that it is the polysaccharides of the ground substance, but that does not mean in terms of functional analysis I should put the cell first, and of this we know extremely little. Dr. Dempsey, what could we do with the cellular elements? You see, we talk about ground substance most of the time, or collagen, or elastin; there is almost never any talk about the cells. I don't know whether a fibroblast, which produces elastic fibers, could be treated with present methods so as to distinguish it from a fibroblast which produces only reticulum or collagenous fibers?

Porter. I don't think the distinction between the cells is as important as determining what influences their work in producing collagen, or what influences their differentiation This can be studied quite satisfactorily with the methods of tissue culture. By isolating the cells and getting them into environments that may be controlled, we can bring all sorts of factors to bear on them. In that way, I think a lot will be learned about what goes into collagen, and how the cell which produces it may be controlled. From the standpoint of the clinician, or the person interested in diseases of connective tissues, I think this would have a great deal of significance.

Holbrook. Very much so

Meyer. If you grow fibroblasts from the area of the aorta, do they produce *in vitro* elastic tissue?

Fell. I don't know Elastic fibers have been described by Bloom, I believe.

Dempsey In very old cultures, was it not? They grow very slowly as compared with collagenous fibers.

Fell Yes, very little has been done. I think it would be interesting to see how elastic cartilage differentiates, too

Dempsey. There is another kind of question that comes up here I think you remember Lansing reporting that the amino acid composition of elastin from the aortas of old animals, has a different amino acid composition from that in young animals, especially a much higher glutamic acid composition. The question immediately arises, does the elastin formed by the fibroblast in the old animals appear to be chemically different from that which is formed in young animals, or is the elastin, once formed, then chemically modified as the animal grows older?

Meyer. That is a very interesting question, because a year or two ago Partridge showed that the enzyme, elastase, splits elastin with the liberation of aspartic acid. It occurs in the pancreas, but

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where else I don't know. But it may indicate that some change of this nature does occur *in situ* in the tissues

The question is whether people interested enough to do this type of work can be found. In biochemistry, for example, a lot of energy is devoted to intermediary metabolism, and one laboratory competes with another to be the first to come out with something If they would take up the study of connective tissues, they could very fruitfully go into a field in which there are perhaps only three or four laboratories working in the whole world There are also too many people working on purine and pyrimidine metabolism, as well as on cancer.

Porter: You ought to put together a compendium of problems for chemists who cannot stay out of each other's way.

Meyer. I am sure that applies to histology also

Dempsey. Connective tissue is just the stuff that is in between the interesting things, to most histologists

Holbrook: I think it is important, and we have really never said so before in a conference. There are a number of problems that are approachable, with methods fairly well worked out, but we lack people to do them.

Dempsey. I think that is true in every one of the fields we have touched on today.

Travell: It is lack of interest.

Meyer: It is amazing to me that when the government tries to find out what can be done, there is apparently no adequate response from the medical schools For instance, there was an inquiry from Washington to find out whether we could do something on arthritis and metabolic diseases, in order to present money requests to Congress However, with the exception of attempting to find a cure for arthritis, there was a dearth of information available as to what might be done.

Holbrook: They took a census of all the medical schools to find out, first, what they were doing in the field of rheumatic disease, or in connective tissue problems, and second, if they weren't doing anything, whether they were handicapped by a lack of funds, and in that case would wish to apply for a grant. You would be surprised how many of the schools came back with just two answers No, they were not doing anything, and no, they did not wish to do anything.

Dempsey: Or didn't know of anything to do

Holbrook: That's right I saw that answer from more than a dozen class A medical schools — just two words, "No, no"

Angevine: I believe there will be some improvement in this direction in view of the fellowships being awarded by the American Rheumatism Foundation. This was started three years ago. The first year there were relatively few highly qualified applicants, the second year showed improvement, and this year there were many fine applicants. You know how slowly the whole field moves, so I am not at all discouraged by what appears to be a shortage of workers in this field at the present time.

Holbrook: There will certainly be more people.

Angevine: But it will take a good many years. I believe more effort should be made to steer applicants in the proper direction, or to the proper person.

Holbrook. They move slowly, but it is at least considerably better than it was twenty-five years ago. I think the fellowships have helped We are now in a situation where financial help is coming from at least three groups: the National Science Foundation; the Public Health grant-in-aid, which is a project type of grant, and the Institute of Arthritis and Metabolic Diseases. There are also the grants from the Arthritis and Rheumatism Foundation for their research fellows, so that actually, within the last three years, the three agencies I have mentioned have made research fellowships available in this field. It hasn't begun to mature yet, but I feel as you do, Dr. Angevine, that the quality of the men that we saw this year was tremendously improved over what it was three years ago

Angevine The young fellows that Dr. Hass has with him are practically all Public Health Service Fellows

Holbrook Yes, they are.

Angevine. That started about two or three years ago.

Holbrook. That's right, so I think we ought to make a real effort to steer some of these youngsters to the people who have more ideas and problems than they have people to work on them.

Angevine. Yes. I think most applicants possess ability and enthusiasm, but very few young men know what they want to work on, and often think that something might be an opportunity for them when actually it is not

Holbrook It is scarcely a concern of this conference, but nevertheless it is something which we hope will materialize.

Dempsey: One thing that seems to have developed during our discussions in the past is that one effect of cortisone on connective tissues is to inhibit fiber formation; that is, collagenous fiber formation. Is it specifically known that cortisone does or does not inhibit

the formation of the other materials made in the connective tissues under the influences of the fibroblast, namely, elastic fibers and the ground substances?

Meyer. The production of ground substance is decreased

Porter: It seems as though the whole protein metabolism, the protein synthesis of those cells, is inhibited

Dempsey: That is what I was getting at. Are the cells stopped in their tracks, or is merely one function stopped, whereas another function may be going on?

Meyer. Dr. Raffaele Lattes, an experimental pathologist at Columbia, has gone into this problem with Dr. Ragan They have studied one approach, or one phase of this cortisone problem in wound healing, and there it can be shown, that while the first phases of the wound healing proceed uninhibited by cortisone, from the third day on migration of the cells into the area is inhibited Oxidized cellulose injected into the area, despite the continuation of the cortisone, is chemotactic for macrophages That suggests one phase of the inhibition of cellular metabolism, whatever it is, as the production of an acidic polysaccharide, not protein Naturally, it does not mean it is not a protein, because in order to produce these substances, you obviously need protein first, namely, the enzymes of the cells

[illegible] -dure with collaboration [illegible] believe I do not think, [illegible] he mechanism of cortisone. It is, perhaps, only a minor phase of the problem in general

Holbrook: Most of those effects have been shown with very unphysiologic doses, if one can call them that, Dr Meyer They have been huge doses

Meyer: Of cortisone? Obviously that is true, that is, this is not physiologic One could not show it in a normal animal with a physiologic dose of cortisone, it won't do anything But this is one way of getting at the mechanism by which cortisone affects connective tissue cells.

Holbrook. I was not aware of the fact that the whole ground substance had been studied in relation to cortisone

Meyer. Not the whole; there are plenty of problems left

Holbrook. But the question Dr. Dempsey asked

Meyer. Oh, yes, that has been studied by the group in our institution It has been studied in other places as well There is quite some literature on it

Holbrook: The question of fibroblasts in wound healing, I know, has been done many times.

Dempsey: The elastic fibers should be studied, I think, after cortisone I don't think anybody knows anything about the formation of elastic fibers, with or without cortisone. They just grow.

Holbrook. Does anyone wish to add anything to that?

Meyer: I don't know, but I should like, as the last thing, to mention the interaction with parenchymatous organs and with the organism as a whole. I personally do not know what to do about it, but I feel that it is really a very challenging problem.

Porter: It is difficult to approach, also, because the connective tissue is so diffuse, and not like the parenchymatous organs of the body, where the cells are well defined and there is good histology.

Holbrook· Be a little more concrete, Dr. Meyer.

Meyer: Does the functioning of the parenchymatous structure depend on the connective tissue stroma? How far does the functioning, let us say, of liver cells, or the functioning of the hair follicle, or any such structure, depend on the supporting structure? What is the interaction, or is there no interaction? I was thinking especially of the balancing of the problem of invasive influences That comes into it. I have seen so many papers which refer to Dr I. Gersh and the depolymerization of the ground substance I have seen quite a few papers which employ the same terminology and use this hypothesis for further argumentation I wish another expert in the field would work on this problem. I don't see that there is a possibility that the substances are depolymerized; I do not think he is right. It would make no difference whether or not they were all polymerized; they would not have enough groups liberated to make an effect I do not doubt his increase in PAS (Periodic-Acid-Schiff) staining, but I doubt his explanation.

Porter: Did he put it out as any more than a theory?

Angevine. Just a proposal.

Meyer. What I object to is his indiscriminate use of the word "depolymerization." I think it is one of our most important functions to keep the concepts as clear and clean as possible We should not confuse them by accepting hypotheses which are contrary to established facts

Travell The hypothesis so often comes to be quoted as fact, though, in transmission.

Angevine: You discussed that in considerable length in your review of hyaluronic acid.

Meyer: No, it hadn't appeared at that time.

Angeline: But I mean, several years ago, didn't you discuss depolymerization?

Meyer: Yes.

Angeline: Would it be all right if you used it in reference to hyaluronic acid?

Meyer: I don't know. Dr. Gersh has not demonstrated that there is depolymerization of hyaluronic acid He has an increased PAS stain This can mean, since they are not small molecular weight substances which would wash out, that this is another substance which hasn't been there before at all; or it can mean that there is more of a substance which was there before, but in smaller quantity; or probably there are some more possibilities The same enzyme which depolymerizes collagen cannot also depolymerize hyaluronic acid.

Holbrook. I should like to thank all of you for your participation

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